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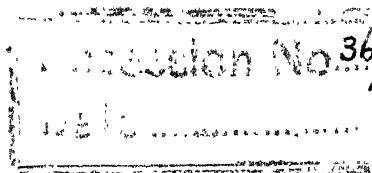
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VOLUME 52

SEPTEMBER, DECEMBER, 1931



THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY  
PHILADELPHIA



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# STUDIES ON THE COMPARATIVE HISTOLOGY OF THE DIGESTIVE TUBE OF CERTAIN TELEOST FISHES

## II. A MINNOW (*CAMPOSTOMA ANOMALUM*)<sup>1</sup>

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### SIX FIGURES

#### AUTHOR'S ABSTRACT

This paper presents a study of the normal histology of the digestive tract of an herbivorous teleost, the minnow *Campostoma anomalum* (Rafinesque). The tunics of the buccal cavity, pharynx, esophagus, and intestine are described; particular attention is given to the mucosa because of the specializations occurring in that coat, such as taste buds, goblet cells, callous pad, etc. Because of the very decided anatomical and histological differences existing between the anterior and posterior regions of the pharynx, due to the presence of a callous pad and pharyngeal teeth, it has been deemed advisable to consider the pharynx as divisible into an anterior and a posterior region. Thyroid tissue was found in the submucosa of the anterior pharynx. No gastric epithelium was demonstrated, the 'intestinal bulb,' the only enlargement of the coelomic portion of the digestive tube, being lined with epithelium presenting only minor differences from that found in the coiled tubular intestine.

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<sup>1</sup> Studies from the Department of Zoölogy, The University of Nebraska, no. 168.

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### INTRODUCTORY

The present study is a description of the histology of the digestive tract of a minnow, the stone-roller, *Campostoma anomalum* (Rafinesque). This fish was chosen because of its uniqueness, being a representative of the almost entirely herbivorous genus, *Campostoma*. Some histological work has been done on the family Cyprinidae to which the stone-roller belongs, but little on *Campostoma*. According to Yung ('99), the following men have worked on the cyprinoid group, particularly on *Cyprinus carpio* and *Tinca vulgaris*: Bischoff ('38), Valatour ('61), Langer ('70), Waalewjin ('72), Biedermann ('75), Edinger ('77), Luchhau ('78), Garel ('79), Cataneo ('86, '87), Decker ('87), and Oppel ('96).

The name of Pictet ('09), who also worked on *Cyprinus carpio* and *Tinca vulgaris*, as well as three other European species, may be added. Kraatz ('23) made a study of the food, and in 1924 published a paper on the intestine of the minnow or stone-roller, laying special stress on the development of the coiling. His work did not cover the anatomical

features of the mouth, pharynx, and esophagus. He noted, however, a simple columnar epithelium with goblet cells in the intestine.

In the author's study several specimens were used, one for anatomical, others for histological studies. Three had been fixed imperfectly in formalin, and the rest in Bouin's fluid; these last were the ones principally worked on. Preservation was in 80 per cent alcohol. The following stains were used: Delafield's haematoxylin, Heidenhain's iron-alum haematoxylin, eosin, methylene blue, and Mallory's triple connective-tissue stain. Of the last, both solutions were used on some sections, while only one solution was used on others. Sections were transverse, longitudinal, and occasionally tangential. The thickness varied from 2.5 to 10  $\mu$ , but the first made very unsatisfactory sections. In general, about 7.5  $\mu$  was the best thickness. The paraffin method was used throughout.

The writer wishes to express her appreciation to Dr. Irving H. Blake, of The University of Nebraska, under whose supervision the work was conducted, for encouragement and aid throughout the study; and also to Mr. R. K. Kinsey and his assistants, for assistance in the preparation of the fixatives and stains used.

The following parts of the digestive tract will be considered: buccal cavity, pharynx, esophagus, intestinal enlargement or bulb, and intestine proper. There is no true stomach in the stone-roller, but there is an anterior enlargement of the intestine, which corresponds in position to the stomach of other fishes.

The parts of the tract were measured in several adult specimens. In general, it was found that the length of the intestine alone was several times (up to more than six) the length of the body of the fish; this is in accordance with the work of Kraatz ('24). The following measurements are typical:



	<i>Cm.</i>
1. Buccal cavity,	.9
2. Anterior pharynx,	.6
3. Posterior pharynx,	.35
4. Esophagus,	.3
5. Intestinal bulb,	2.2
6. Intestine proper,	52.0

#### BUCCAL CAVITY

The buccal cavity extends from the lips to the first gill slit, or to the pharynx. It begins as a small, narrow cavity, roughly about 3 or 4 mm. wide, and quite rapidly widens, reaching its maximum width just at the anterior boundary of the pharynx. Roughly, it can be divided into the roof and the floor. There is little difference between the two regions other than that the floor contains the rudimentary tongue, and the roof a large triangular fold. The epithelium of both regions is lifted up in a series of low longitudinal folds, the folds being more pronounced at the sides of the cavity.

The following are the histological divisions of the mouth: *a*) mucosa (epithelium, basement membrane, and stratum compactum); *b*) submucosa.

#### *Buccal mucosa*

*Epithelium.* The epithelium of the buccal cavity possesses these elements: undifferentiated epithelial cells, cornified desquamating cells, mucous cells, and taste buds. Some of these elements are sharply localized.

The ordinary undifferentiated epithelial cells are present almost everywhere, around taste buds and mucous cells, as well as forming the basal layer of the epithelium. These cells, when crowded very little, have a regular, polygonal shape. They are found thus in the anterior mouth, as well as on the lips. In the posterior part of the mouth, this regularity of the cells is lost because of the great pressure of the goblet cells and taste buds. The definite cell outlines are also lost in the posterior part of the mouth; the nuclei, however, are in general smaller than those of the surface, and are more rounded. The cells at the surface, while still

typically regular, begin to show a slight tendency to flatten out (fig. 1).

The dead, desquamating cells are not common to all parts of the mouth. They are found only within the first millimeter of the buccal epithelium, being restricted to a median ventral and median dorsal zone. On each side of this zone, the ordinary buccal epithelium is present. This median zone is characterized by especially large polygonal epithelial cells,

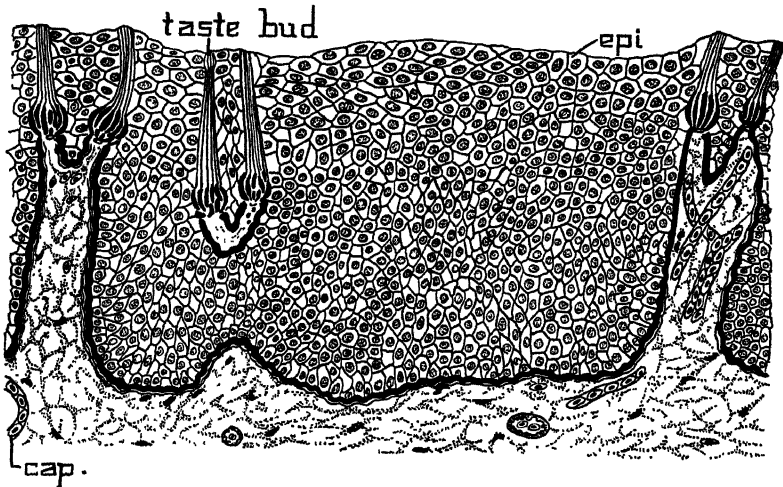


Fig. 1 This drawing of a transverse section of buccal mucosa and submucosa shows the type of tissue which is found on each side of the rudimentary tongue. Taste buds are present on the slender papillae of vascular submucosa. Capillaries (*cap.*) are seen in the vicinity of the taste buds. The epithelial cells are shown to be larger and differently oriented at the surface than at the base of the epithelium (*epi.*).

topped with the layer or two of dead cells. The dead cells are flattened, and lack the full, distinct nucleus of the underlying living cells. The cells below the desquamating layer have large nuclei and nucleoli. The basal portion of this region is marked in cross-section by a number of long, slender, finger-like projections of submucosa. These finger-like projections are lacking in longisection, the base of the epithelium appearing flat. This holds true for both the floor and the roof of the mouth. This type of epithelium is found in only

one other place in the alimentary tract—in the dorsal callous pad of the posterior pharynx. On the tip of the tongue, this structure is uniform in thickness and pierced by a number of thin, very evenly spaced submucosal folds. In the roof of the mouth this specialized epithelial structure can be divided into three zones—a median zone and two lateral zones, one on each side of the median zone. The lateral zones

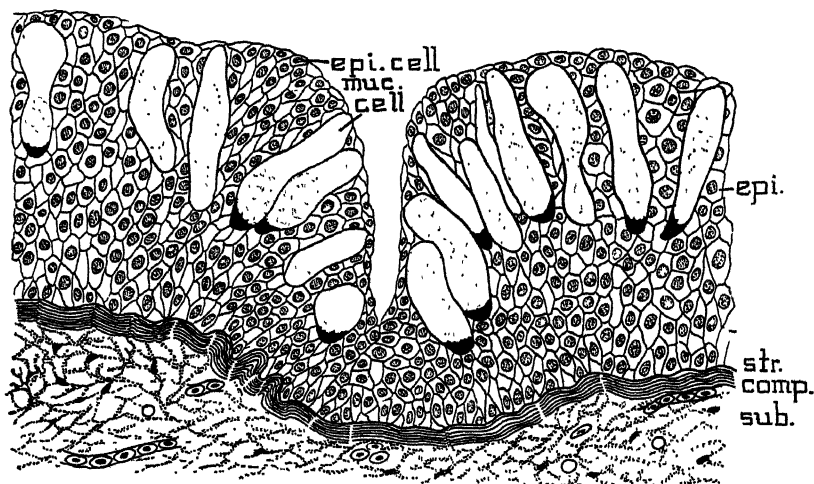


Fig. 2 Drawing of transverse section, showing epithelium, stratum compactum, and submucosa, taken posterior to figure 1. It shows the comparative size of undifferentiated epithelial and specialized mucous cells. A stratum compactum is shown in greater detail here than in the preceding drawing, appearing as a fibrous band rather than a solid black line. It has inconspicuous vertical interruptions. A very thin basement membrane underlies it. Capillaries are relatively common in the submucosa. *epi.*, epithelium; *epi.cell*, epithelial cell; *muc.cell*, mucous cell; *str.comp.*, stratum compactum; *sub.*, submucosa.

are considerably thinner than the thick median zone. The base of the three zones is pierced by numerous very thin longitudinal submucosal folds (fig. 3). This area corresponds roughly to the papillary region described by Pictet ('09) for *Tinca vulgaris*.

Mucous cells are present on and about the lips as well as in the digestive tract. Their number is somewhat limited in the anterior part of the mouth, but increases as one goes

posteriorly. No goblet cells were evident in the specialized epithelial structure corresponding to the papillary region of Pictet.

Behind this structure is found a fold which is transitional in the sense that it has epithelial cells of the undifferentiated type, goblet cells, and taste buds. Behind this fold the goblet cells and taste buds gain prominence. The goblet cells become

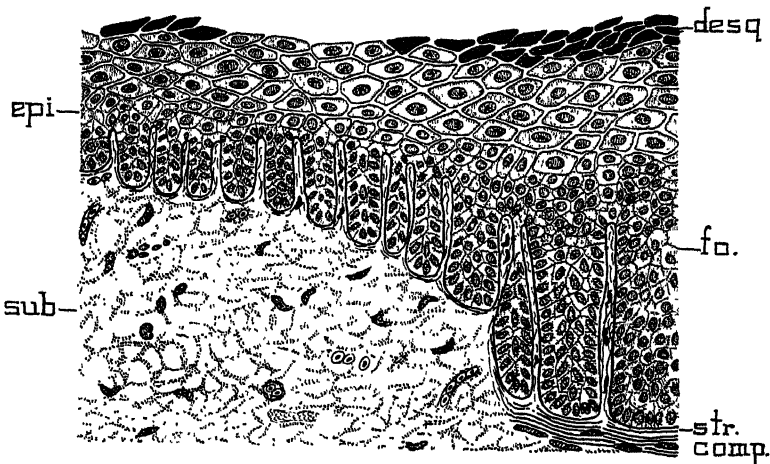


Fig. 3 Drawing of a transverse section of the somewhat calloused epithelial (*epi.*) structure of the first millimeter of the roof of the mouth. On the right is seen a part of the thick central zone and on the left, the thinner lateral zone with very large and regular epithelial cells. A surface layer of dead cells (*desq.*) is evident. Since this is a transverse section, it shows the uniform, narrow, longitudinal folds of submucosa (*sub.*) as finger-like projections into the epithelium. The stratum compactum (*str.comp.*) is quite thick under the central zone. *fo.*, folds.

so numerous that they give the outer half of the epithelium a blue color, while the basal half is yellow because of the number of epithelial cells; Mallory's stain brought this out very clearly.

Posterior to the transition zone may be found mucous cells which are somewhat atypical. Their nuclei, instead of being flattened at the base as is normally the case, are round. Also, they are found in a finger-like projection of the base of the cell. Their nucleoli stain very distinctly. The upper part

of the mucous cell is an enlarged sac, sometimes showing a reticular arrangement of the contents. Sometimes the number of mucous cells is so great that the epithelial cells are crowded away from the surface.

The organs (fig. 1) referred to both as sense and as taste buds (by Pictet) Herrick ('02) definitely states to be taste buds, even though found outside the body. In *Campostoma* they are flask-shaped. Pictet, in studying these organs in other species of Cyprinidae, found that there were two types of cells: the inner sensory cells and the outer enveloping nutritive cells. Both types, but the nutritive cells in particular, were characterized by elongate nuclei. In *Campostoma* the outer cells are so clearly arranged that the structure of the inner cells is difficult to determine. Moreover, the staining reaction of the nuclei of the outer cells is so intense that quite often the bulbous part of the taste bud appears as a dark mass of nuclei. The nuclei of the outer cells are long and narrow. The protoplasm of each outer cell is drawn out into a long narrow process reaching to the surface of the epithelium (fig. 1). The technique employed did not satisfactorily reveal the structure of the inner cells which were present.

The buds are quite plentiful about the lips, but not so numerous as in the anterior parts of the buccal cavity. They are a common element in the mucosa after the first millimeter of buccal epithelium. One very interesting fact about them is that they are always found on a slender finger-like papilla. Often two buds are present on the same papilla. Pictet, working on the tench and the carp, found three and even five buds resting on one papilla.

*Basement membrane.* A basement membrane, though somewhat hard to distinguish, is present as a thin fibrous sheet between the epithelium and the stratum compactum. It is blue when stained with Mallory's connective-tissue stain.

*Stratum compactum.* This layer is present in the mouth as a clear, wavy, uniformly staining band of fibers. In a few places, connective-tissue cells may be found between the fibers.

*Buccal submucosa*

Below the stratum compactum lies the submucosa, which is made up of rather fine strands of areolar connective tissue, capillaries, larger blood vessels, fat, and connective-tissue cells. As one goes away from the mouth, the fat cells increase in number, crowding the connective-tissue elements.

The premaxillary bones, underlying the submucosa, have many slightly wavy collagenous fibers about them, as well as numerous capillaries.

In the tip of the rudimentary tongue and in the lower lip is a membrane composed of large, uniform, very clear, stratified cells and traversed by many vertically directed collagenous fibers. In the tongue, this membrane is found to occupy most of the space in the submucosa.

The cone-shaped papillae on which the taste buds are borne are fairly numerous. In them are found capillaries, connective tissue, and nerve fibers. The technique employed did not differentiate the nervous elements, but the work on taste buds by others substantiates the statement.

## PHARYNX

The division of the pharynx into an anterior and a posterior region is very marked, both anatomically and histologically. The anterior region extends from the first through the last gill slit; the posterior includes the region of the ventral pharyngeal teeth and a dorsal callous pad. These layers are present in the two divisions of the pharynx: *a*) mucosa (epithelium, basement membrane, and stratum compactum, the last in the anterior region only); *b*) submucosa, greatly reduced in the posterior region; *c*) muscularis.

The surface of the roof has a wrinkled appearance, and just in front of the callous pad of the posterior pharynx are a few short, flattened folds. The floor also has a few folds, but of a different type from the hardened ones of the roof. The amount of uninterrupted surface is greater in the roof than in the floor because the gill slits are lateroventral in position.

*Anterior pharyngeal mucosa*

The mucosa of the anterior pharynx consists of the following layers: epithelium, basement membrane, and stratum compactum.

*Epithelium of the roof.* The buccal epithelium continues into the pharynx. Large mucous cells make up the upper one-third to one-half of the thickness of the epithelium, while crowded, misshapen epithelial cells whose boundaries are very indistinct make up the rest. The mucous cells have a variety of shapes, being round, sacciform, finger-like, or a combination of these. Two features may be noted: first, the 'reticulate' effect produced by the mucus and, secondly, the type of nucleus, which is usually basal, but rounded rather than flattened against the base of the cell. A distinct nucleolus and a few coarse masses of chromatin material may be seen. Stratification of the cells is quite common except between the folds in some of the crypts.

A mass of discharged mucus may sometimes be seen on top of the epithelium and in the crypts, being often as thick as the epithelial layer itself. This condition was found in only a few sections, and may be due to poor fixation or to fixation during a certain stage of secretion. The mucous cells had broken down in some places, discharging their mucus into the colloidal mass referred to.

In the last millimeter, just in front of the dorsal callous pad, are a series of low, flattened folds, converging toward and terminating before the pad. Their crypts are of approximately the same width at top and bottom; they are lined with both mucous and ordinary epithelial cells, the former being found usually at the base. The mucous cells are not very plentiful at the top of the crypts. At the sides, they are localized, and may sometimes form a small secondary crypt. Elongate and somewhat spindle-shaped epithelial cells are almost as plentiful along the crypts as the goblet cells.

Taste buds are present in the roof, but appear to be more numerous in the floor and sides.

*Epithelium of the floor.* The floor is about three times wider at the first than at the last gill slit. The epithelium is usually thickest along the median line, thinning suddenly to a few layers of flattened epithelial cells at the gill slit.

The uniform, though somewhat flattened, epithelial cells appearing where there are few mucous cells readily fall into three divisions: the horizontally oriented surface cells, the vertical or oblique middle layer, and the basal division which is perpendicular to the stratum compactum. When taste buds and goblet cells are present, this arrangement is disturbed slightly.

The taste buds of the pharynx are like those of the buccal cavity. They are abundant, and are situated on top of the papillae of submucosa. The stratum compactum is very evident everywhere except just beneath the taste buds.

*Basement membrane.* A thin membrane is present just above the stratum compactum. The details of structure could not be made out.

*Stratum compactum.* The wavy, hyaline stratum compactum of the anterior pharynx is slightly thinner than that of the mouth. The underlying submucosa raises it up to form a number of papillae which bear taste buds. No invasion of the stratum by capillaries was noted here. Neither were the fibers of the stratum so apparent as in the buccal cavity.

### *Submucosa*

The pharyngeal submucosa at first differs very little from that of the oral cavity, being areolar, very vascular, and heavily impregnated with fat. Posteriorly, the spaces between the fat cells become filled with striated muscle fasciculi. The increased amount of muscle finally reduces the submucosa to a narrow layer of connective tissue which is slightly deeper in the papillae. Just between the last pair of gill slits, this layer is thinned down to scattered connective-tissue cells and occasional fibers. Thyroid tissue was found in the submucosa of the anterior pharynx.



*Muscularis*

The amount of muscular tissue is not as great in the anterior as in the posterior region. It appears first in the form of longitudinal fasciculi at the base of the buccal submucosa and continues into the posterior pharynx. At first, there are a great number of fat cells, then between them begin to appear isolated and rather small fasciculi, some longitudinal and others transverse. Between the last pair of gill slits the muscle is separated from the stratum compactum by a very thin layer of submucosa. The muscle here is more compact, being in definite bundles. A change is very evident in the pharyngeal musculature between the first and last gill slits; the fat cells grow smaller, while the number of fasciculi about them is augmented. Numerous capillaries are present. The figure (fig. 5) shows a portion of pharyngeal tissues and is fairly typical of the region between the fourth and fifth gill slits.

The posterior pharynx is characterized by the following anatomical features: first, a flat, oval, callous pad, about  $2.5 \times 3.5$  mm., occupying the roof; secondly, a wedge-shaped strip of mucosa on each side of the pad; and, thirdly, the paired inferior pharyngeal bones with their four pairs of teeth partially embedded in the ventral submucosa.

Sectioning of the floor is particularly difficult because of the pharyngeal teeth and bones. Removal of the hardened structures is equally difficult.

A macroscopic examination shows these parts: first, the flat, crescent-shaped stretch of epithelium supported by the pharyngeal bones; secondly, posterior to this crescent-shaped 'plateau,' a rough, longitudinal median ridge. On each side of this are four pharyngeal teeth alternating with transverse ridges of epithelium. The latter are higher than the teeth.

*Posterior pharyngeal mucosa*

The mucosa of the posterior pharynx differs from the regions anterior to it by the absence of the stratum compactum.

*Epithelium of the roof.* The extremely thick layer of desquamating cells on the pad stains a brilliant red with Mallory's triple stain. The top of the pad is narrower than the base, and the thickness is greater at the sides than at the middle. About twenty-five extremely slender submucosal folds penetrate halfway up the pad, being taller at the sides than at the center.

The cells of the pad are practically identical with those found at the tip of the tongue, being very large, polygonal, and oriented in the same manner. Very careful observation shows frequent intercellular cytoplasmic bridges.

In the anterior part are present a few short, wide taste buds within the epithelium.

On each side of the pad are a few low folds, their crypts lined with large sac-like mucous cells. Lateral to these are the wedge-shaped folds which differ markedly from the pad, possessing none of its large, polygonal, epithelial cells. No taste buds were visible, although several submucosal elevations were present. The tops of the folds were somewhat flattened. The stratification of slender, long, spindle-shaped epithelial cells results in a thick epithelium. Mucous cells are present, but desquamating cells are lacking.

*Epithelium of the floor.* The epithelium of the anterior pharynx gradually merges into that of the 'plateau,' thickening slightly. The epithelium is thickest at the point of termination of the 'plateau,' and is composed mainly of long, slender, almost cylindrical, stratified epithelial cells, the orientation of which is vertical except where disturbed by taste buds, mucous cells, or submucosal papillae. The plateau ends abruptly, giving way to tall folds, some of which branch. The crypts between them are lined with mucous cells from one to four layers deep. Unmodified epithelial cells are present at the base. Some of the highest folds have a number of epithelial and comparatively few mucous cells at the tip.

In one specimen the 'plateau' was covered by a fairly thick layer of flattened cells which had a curiously 'empty' appearance, being without nuclei. Some of these cells were

sloughing off into the pharyngeal cavity. They stained a pale blue with Mallory's stain. The surface layers of the corresponding dorsal region had the same peculiar appearance. The condition may have been due to poor fixation or other causes, since only one specimen showed the peculiarity.

*Basement membrane.* The fibrous elements of the submucosa which run up to the epithelium obscure the basement membrane, which is a very thin layer. A stratum compactum is absent from both the ventral and dorsal posterior pharynx.

### *Submucosa*

The areolar tissue is invaded by numerous striated muscle fasciculi. The submucosa is thicker on each side of the pad than under it, because the muscle invasions are less heavy in the former place. A cross-section shows the pad resting on a very narrow strip of submucosa, with so many fasciculi that in a few places some of them press against the epithelium. The base of the pad is penetrated by a number of high, uniformly spaced, and very slender folds of submucosa.

Wherever a bone is found, there is some fibrous tissue. The bones themselves are not an element of the submucosa, but sometimes are found there, especially in the anterior pharynx.

### *Muscularis*

The amount of submucosa is considerably limited by the invasion of both longitudinal and transverse striated fasciculi. Below this mixed zone is a band of connective tissue followed by transverse fasciculi, which in their turn are followed by longitudinal muscle. There are about five or six transverse bands separated by a strip of connective tissue. This is particularly evident in the dorsal pharynx. Fat cells are scarce, but capillaries are abundant around the fasciculi.

### ESOPHAGUS

The esophagus is a small, uniformly thick tube about 3 mm. long. It is characterized by a large, anterior, longitudinal,

dorsal crypt or pocket which is bordered by folds on each side. In addition, there are about ten tall longitudinal folds between which are present two or three small folds. A pneumatic duct leading to the second chamber of the air bladder is a dorsal diverticulum of the esophagus. The four layers present in the esophagus are: *a*) mucosa, *b*) submucosa, *c*) muscularis, and, *d*) serosa. A true stratum compactum is absent.

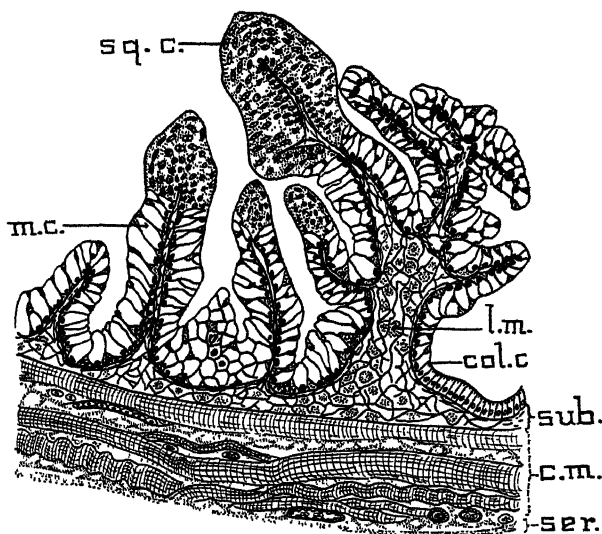


Fig. 4 This is a cross-section of the midesophagus, showing two major folds, their tips covered with stratified epithelium and their sides with mucous cells, a number of smaller folds, and on the right a small part of the dorsal crypt mentioned in the text. *col.c.* are the columnar cells which pass over into the lining of the pneumatic duct. The nuclei of these and the mucous cells are basal. Striated longitudinal fasciculi (*l.m.*) are found within the meshes of the fibrous submucosa (*sub.*). The circular muscle (*c.m.*) is just above the serosa (*ser.*).

### *Mucosa*

*Epithelium.* In the anterior part the mucous cells are very numerous, but are rare at the entrance of the esophagus into the intestinal bulb. They line most of the large anterior dorsal crypt mentioned above. A small portion of this pocket is shown (fig. 4).

The ten or so major longitudinal folds are characterized by a thick layer of stratified epithelial cells covering their tips, and a number of large, distended mucous cells on the sides. Taste buds are present on some of the tips.

The smaller folds between the major ones do not show this differentiation. They are covered entirely with mucous cells, with the exception of some of the taller folds, which may

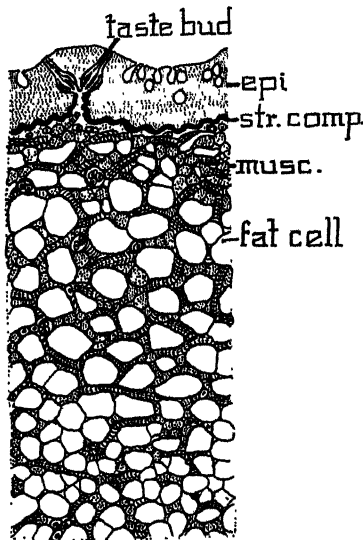


Fig. 5 Low-power drawing of section of anterior pharynx. The epithelium (*epi.*) has many squamous and fewer goblet cells. Two taste buds on one papilla are fairly common. A definite stratum compactum (*st.comp.*) is present, but the submucosa is reduced. The lower portion of the figure shows the great amount of fat present between the fasciculi.

occasionally have a small number of stratified epithelial cells at their tips.

The pneumatic duct, just at the point of departure from the esophagus, is characterized by clear columnar epithelial cells with basal nuclei.

The end of the esophagus is marked by a 'mixed' epithelium, showing the ordinary stratified epithelial cells and a new type, a single layer of cylindrical cells. The latter are rather wide columnar cells, staining an even color through-

out. Moreover, they do not show the tendency to shrink away from the neighboring cells, as do the taller, more slender, intestinal epithelial cells. The cylindrical cells are topped by a cuticular border or top-plate, while the ordinary stratified cells show no such structure. Various combinations of these cells may be found. Some of the folds may be formed of one type of cell and the base of the crypts of another, while other folds may have the simple epithelium on one side and the stratified on the other. Pictet mentions a slightly different type of mixed epithelium in four cyprinoids.

*Basement membrane.* A very thin basement membrane is present. A true stratum compactum is absent, although in places the submucosa is so fibrous and so close to the epithelium that it bears a very strong resemblance to this structure.

#### *Submucosa*

As in the pharynx, the submucosa is considerably reduced by the amount of invading muscle, but is thicker at the base of the folds and at the entrance of the esophagus into the intestine.

#### *Muscularis*

Striated muscle is the only type found in the esophagus. It forms a very thick coat. The dorsal wall, adjacent to where the pneumatic duct leaves the esophagus, is thin as compared with the ventral and lateral walls, and also shows a reversal of the muscle layers described below. Around the duct below the narrow submucosa is the inner circular muscle layer, and below that is the outer longitudinal one. A small amount of connective tissue separates the two.

The wall of the rest of the esophagus is considerably thicker. Longitudinal muscle is present in the meshes which are extensions of the fibrous submucosa. The fasciculi are separated from their neighbors by a space surrounded by a few collagenous fibers. Dawes ('29) found longitudinal fasciculi in the submucosa of the plaice. The outer, circular layer is more closely packed than the inner longitudinal, the fasciculi being closer together and in larger bundles.

The posterior boundary of the esophagus shows a very well-defined submucosa which is also invaded by small bands of striated circular muscle which increase in diameter and number as they approach the serosa. In this region the pneumatic duct is shown as a separate structure.

### *Serosa*

The free part of the esophagus shows a fairly thick serosa, which is composed of connective tissue, capillaries, occasional larger blood vessels, and a surface layer of very flat peritoneal cells. The serosa of the esophagus is thicker than that of the intestine; this, however, may be due to the greater contraction of the former organ, especially in fixed material.

### INTESTINE

A true stomach is absent in *Campostoma*, but its place is taken by an enlargement which for convenience has been designated as the intestinal bulb. Kraatz, in speaking of this region, calls it the 'swollen part of the intestine,' but makes no effort to make a definite division of it. The choledochal duct enters the posterior part of the enlargement. The long intestine behind the enlargement is nearly uniform in thickness. It is a very slender tube wound around the air bladder. Kraatz has made a very careful study of its coiling. The diameter of the posterior part of the intestine is somewhat smaller, especially near the anus. The intestine proper possesses the same tunics and almost the same cellular elements as the bulb.

The prominent zigzag folds of the mucosa can be seen readily through the semitransparent wall of the intestinal bulb. The height and complexity of the folds decrease as the tubular region of the intestine is reached. Uniformity in thickness characterizes the upper and lower parts of these folds, many of which unite, producing a branching effect in which the branches have the same width as the main fold. The turns formed by this branching or junction of folds are angular in the anterior part of the bulb. Toward the pos-

terior part, the angular folds have given way to less angular ones.

The following layers are found in both the bulb and the intestine proper: *a*) mucosa (epithelium, basement membrane); *b*) submucosa; *c*) muscularis; *d*) serosa.

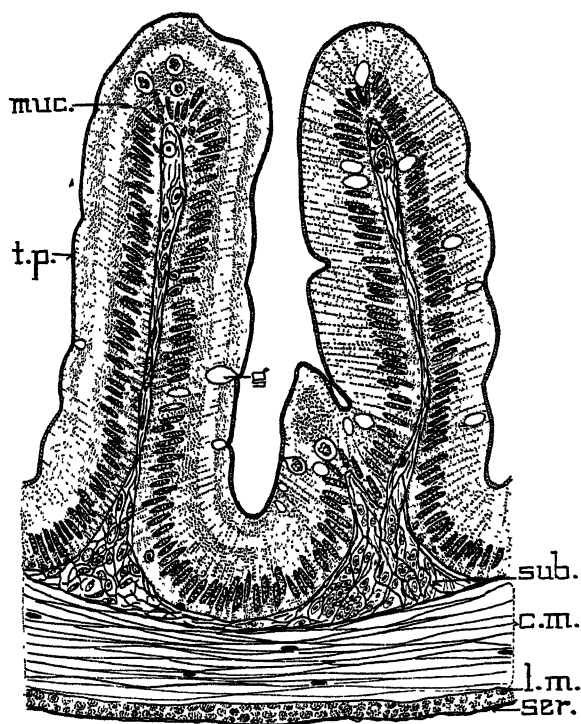


Fig. 6 A typical cross-section of intestine. *t.p.*, striated top-plate; *g.*, goblet cell; *muc.*, mucous (goblet) cells in an early stage of development; *sub.*, submucosa; *c.m.*, circular muscle; *l.m.*, longitudinal muscle; *ser.*, serosa.

### *Bulbar mucosa*

*Epithelium.* The epithelium of the entire intestine, inclusive of the bulb, is simple columnar, the cells being very slender and tall, and resting on a very thin basement membrane, which in turn is supported by a thin sheet of submucosa. The nuclei of the cells are in the basal third, being slender, elongate, and possessed of fairly large masses of chromatin material.



Small wandering cells are quite common in the epithelium.

Mucous cells are not very numerous at first, but are more so in the posterior region. Nowhere in the intestine are they so plentiful as in the mouth, pharynx, and esophagus, nor do they present such a variety of shapes and sizes. The enlarged or mucus-filled portion of the intestinal mucous cell is spherical. The position of the enlargement varies; sometimes it is found at the base, and may occur anywhere between there and the top-plate.

No multicellular glands of the gastric or intestinal types were found anywhere in the bulb or intestine proper.

A distinctly striated top-plate covers the epithelium (fig. 6).

*Basement membrane.* A very thin basement membrane is present, but a stratum compactum is wanting.

### *Submucosa*

The submucosa is present in every fold as a thin sheet of very vascular, fibrous connective tissue. It is thickest just at the bases of the folds. In general, it may be said that the submucosa does not form a very thick layer in the intestine or bulb.

Where the esophagus enters the bulb, the submucosa is much thicker than the submucosa of the rest of the bulb; the fibers are also finer.

### *Muscularis*

Fasciculi of striated esophageal muscle continue for a short distance into the anterior part of the bulb. As they leave the esophagus, they are in several longitudinal and transverse layers, but finally are reduced to two: the inner circular or transverse, and the outer longitudinal layer. They still retain their striated character for approximately a third of the bulb, then give way to smooth-muscle fibers, which constitute the muscularis of the rest of the digestive tract. The circular layer is thicker than the longitudinal. The amount of connective tissue between them is usually small.

*Serosa*

Though there is some variation in the thickness of the serosa in different parts of the tube, it may be said that it is a very thin layer of characteristic simple squamous epithelium backed by connective tissue.

Diffuse pancreatic tissue surrounded with fat cells is found along the length of the entire intestine. The centro-alveolar cells and the zymogen granules of the pancreatic cells show plainly. Whether any pancreatic tissue is present in the liver was not determined, but will be investigated later.

The spleen (not previously described by Kraatz) is found in a loop of the middle intestine, where the loop rests on the large, left lobe of the liver. The dimensions of the spleen are:

	<i>Mm.</i>
Width,	2.5
Height,	3.0
Length,	5.0

There is no differentiation of the posterior part of the tract into the small and large intestine. The tube is uniform throughout, except in the last few millimeters, where it tapers slightly.

*Intestinal mucosa*

The intestinal epithelial folds decrease in height from anterior to posterior end. Those of the intestine proper are much smaller than those of the bulb; they also lack the complicated arrangement described for that organ. The angularity of the folds is lost, but the same elements are present in the two regions: numerous tall, slender epithelial cells, and comparatively few goblet cells.

In some preparations of both the bulb and the intestine, particularly those stained with haematoxylin, three regions are brought out in a layer of simple columnar epithelium. Under the striated top-plate is a fairly narrow band of darkly staining cytoplasm; next comes a thicker, lighter-colored layer resembling mucus; and lastly, the lower half of the cell containing the nucleus surrounded by cytoplasm similar to

that of the first layer (fig. 6). This appearance of the epithelial cells is due to the fact that they are changing into mucus-secreting cells; the mucus first appears somewhere in the middle of the cell and thence spreads to either the upper or lower zones.

The epithelial cells of the last few millimeters of the intestine, where the folds are very low, are slightly different from the cells of the anterior intestine. They are lower and not so slender. In this respect, they resemble the cylindrical cells described in the esophagus. Their nuclei have shifted from the basal part of the cell to the middle and have assumed a more oval shape.

A thin basement membrane underlies the epithelium.

### *Submucosa*

The submucosa of the intestine is essentially the same as that found in the bulb, with the exception of the atypical region where the esophageal and bulbar submucosae meet.

### *Muscularis*

Two layers of smooth muscle, an inner circular and an outer longitudinal one, are found in the intestine proper. The circular layer is slightly thicker than the other. The nuclei of the fibers are elongate and relatively small.

### *Serosa*

In places, the serosa is extremely thin, but ordinarily it can readily be seen. It is a continuation of the serosa of the bulb.

### SUMMARY

The buccal cavity is characterized by the presence of a rudimentary tongue, the anterior part of which resembles histologically the callous pad of the posterior pharynx. An area of the epithelium of the roof of the mouth just above the tongue shows a greater resemblance to the pad than does the tongue. On each side, and posterior to this specialized

area of epithelium, is found the typical buccal mucosa with its numerous mucous cells and taste buds. A basement membrane separates the epithelium from the very definite stratum compactum. A submucosa is present.

For convenience, the pharynx is divided into two regions: the anterior, being the region of gill slits; and the posterior, being the region of the dorsal callous pad and the ventral pharyngeal teeth. The callous pad is topped by a very thick surface layer of dead cells. The base of the pad is raised up by a number of submucosal folds simulating papillae when seen in cross-section. Taste buds and goblet cells are abundant in the anterior pharynx and less conspicuous in the posterior. The submucosa is present in both regions, but is greatly reduced by the invading pharyngeal muscles of the posterior part. Thyroid tissue was found in the submucosa of the anterior pharynx. A stratum compactum is present only in the anterior pharynx. The great amount of adipose tissue between the fasciculi leads to the peculiar appearance of the pharyngeal muscularis.

A dozen major folds, their tips covered with stratified epithelial cells, and their sides with distended goblet cells, characterize the esophagus. Goblet cells attain their greatest development here. Taste buds are present, but not in great numbers. A true stratum compactum is absent, although in places the arrangement of the adjacent connective tissue simulates this structure. The submucosa is limited by the invasion of striated longitudinal muscle, which is regarded as the first layer of muscle. The outer muscular layer is circular in direction. These layers are somewhat disarranged in the region where the pneumatic duct leaves the esophagus. The outermost coat of the esophagus is the comparatively thick serosa.

The transition from esophagus to the intestine is quite abrupt. The enlarged anterior part of the intestine has been designated as the intestinal bulb. The simple intestinal epithelium is composed of many long, slender columnar cells and less plentiful goblet cells, and is topped by a striated

cuticula. A submucosa, the fibers of which are very coarse, penetrates into every fold of mucosa. The anterior third of the bulb has a muscularis composed of striated fasciculi, which soon give way to two layers of smooth muscle: an inner circular and an outer longitudinal. The outer layer is the thin serosa.

#### CONCLUSIONS

The most striking histological features of the digestive tract of the minnow *Campostoma* are the following:

1. The presence of the callous pad of the posterior pharynx, with its thick, surface layer of dead cells, is not common to fishes in general.

2. A true stratum compactum is limited to the mouth and the anterior pharynx. Greene ('12) found a stratum compactum in the stomach, the intestine, and the pyloric caeca of the king salmon.

3. Taste buds are found as far down as the esophagus. They differ from a typical taste bud of higher forms such as man, in that they are flask-shaped and are located on the tip of a high submucosal papilla. They very frequently occur in pairs.

4. A true stomach is lacking, as shown by the absence of any multicellular gastric glands. The intestine is also of a very simple type, lacking multicellular glands. Their place seems to be taken by the mucous cells which are found in the entire tract.

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# CYTOPLASMIC STRUCTURES IN BINUCLEATE OPALINIDS, WITH SPECIAL REFERENCE TO THE GOLGI APPARATUS<sup>1</sup>

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FOUR PLATES (SEVEN FIGURES)

## AUTHORS' ABSTRACT

Three types of cell inclusions are demonstrated within the general protoplasm of the binucleated *Protopolina*, parasites of *Hyla aurea*. These are considered to represent mitochondria, together with associated, synthesized vegetative granules and Golgi bodies, as evidenced by their behavior, morphology, and staining reactions.

The Golgi material is shown to consist of irregularly twisted rods and granules scattered at random in the cytoplasm, but possessing a very distinctive morphology and reaction to different techniques when compared with the mitochondria. No relationship could be detected between these vegetative structures and the cilia, as has been previously described.

These observations have also been extended to a similar study of the cytoplasmic organs of *Nyctotherus cordiformis*, and it has been possible to demonstrate Golgi bodies of a similar appearance within this organism as well as to show again the nature of the basal granules and their relationship to the cilia.

The procedure of identifying the mitochondria elements with great care is recommended as a preliminary means of studying the Golgi apparatus of Protozoa, particularly where osmication techniques are used exclusively.

## INTRODUCTION

Recent investigations on the Golgi apparatus in Protozoa have been made, both in free-living and endoparasitic forms, mainly with the view of revealing some type of structure which may be considered the ancestral homologue of the varied and often complex apparatus found in metazoan cells. Several authors have recorded, with some disappointment, the similarity between the Golgi elements in Sporozoa and those of metazoan germ and embryonic cells, while others, using somewhat capricious techniques, have described some of the specialized cytomicrosomes found only in Protozoa as representing true Golgi elements. A more exhaustive examination whereby the mitochondrial and associated cell bodies were first isolated, as a preliminary to the study of the Golgi apparatus, might have shown that several of the structures described by authors, in ciliates particularly, could not be homologized with the Golgi elements of other cells.

<sup>1</sup> This investigation was carried out with the aid of the Cancer Research and Treatment Fund.



It is perhaps unfortunate that, where the protozoan cytoplasm is specialized in containing vacuolar and flagellar mechanisms, detailed study of the mitochondrial components has not been made before using techniques specific for the Golgi apparatus. Several recent investigators(1, 2) have drawn curiously involved comparisons between the structures they designate as Golgi material in Protozoa and the ciliary and flagellar apparatus, partly because they have failed first to distinguish the mitochondria from the remaining cell inclusions.

Since the Golgi apparatus in the Sporozoa has already been identified by several independent writers(3, 4, 5, 6) as closely resembling that of other types of cells, the present investigation has been made with an example of the Ciliata, where much controversy exists on the nature of the Golgi apparatus, mitochondria, and other cytoplasmic organs.

#### METHODS OF INVESTIGATION

In order to make these studies sufficiently critical, techniques have been chosen which not only demonstrate the Golgi apparatus alone within the cell, but also those which are supposedly specific for other cell inclusions and which fail to reveal the Golgi elements. The ciliates *Protoopalina intestinalis* (Stein) and *Protoopalina hylarum* (Raff)<sup>2</sup> (from *Hyla aurea*), in view of their relatively simple structure involving the absence of permanent vacuolar<sup>3</sup> and complex flagellar mechanisms, were considered suitable material for this investigation. To amplify this study, observations were similarly made on *Nyctotherus cordiformis*, which frequently accompanies the opalinids in the rectum of the host.

For mitochondrial preparations the *Opalina* were fixed in strong Flemming without acetic, embedded in paraffin, and sectioned at 3  $\mu$ , with a subsequent staining in Heidenhain's iron haematoxylin. This technique was supported by intra-

<sup>2</sup> The identification of these species is taken from Metcalf's monograph (see bibliography).

<sup>3</sup> We have not found the posterior excretory bladder and its longitudinal canal to be a constant feature of these forms. In any case, the structure is not comparable to the permanent vacuoles and excretory mechanisms of other ciliates.

vitam staining with Janus green and Janus red. In order to reveal the Golgi apparatus, the organisms were fixed in situ within the rectum or isolated in great numbers and treated with Da Fano's cobalt silver nitrate. The Da Fano preparations of *Nyctotherus* were counterstained with neutral red and in several instances the Cajal silver-nitrate technique was used to confirm the presence and morphology of the Golgi bodies in the ciliates. In repeating the work of King and Gatenby preparations were made with Champy's fluid and subsequent osmication.

The extreme ease with which fixatives penetrate the cell membrane and the sensitiveness of the cytoplasm to irregular osmosis made it possible to detect at once the material which had been poorly fixed or irregularly stained and impregnated. It was considered of utmost importance to supplement the osmic techniques with silver impregnation, because the isolated, easily penetrable organisms became so thoroughly osmicated with the Hirschler technique that bodies other than those of Golgi nature were also revealed.

Photomicrographs, taken with critical illumination and printed from untouched negatives, are used to illustrate the various inclusions revealed by the several techniques.

#### MITOCHONDRIA AND ASSOCIATED INCLUSIONS IN PREPARATIONS TREATED WITH FLEMMING AND HAEMATOXYLIN OR STAINED INTRAVITAM

##### *a. Opalina*

As previously shown by one of us(7), the mitochondria of binucleate *Opalina* are revealed in chrome-osmic preparations as short, deeply staining rods aggregated generally in the endoplasm with a precise longitudinal orientation which is particularly apparent within the peripheral regions of the longitudinal sections of the ciliate. Many hundreds of these characteristic cell bodies may be seen either isolated or in close contact with faintly staining, coarse granules scattered at random in the cytoplasm. The mitochondria invariably lie in close contact with the surface of these often spherical, lightly staining granules, giving many of these rod-like elements a curved contour (figs. 1 and 2).

Occasionally the mitochondria are bifurcate in Y- and V-shaped conditions, suggesting, as Horning states(7), the incomplete longitudinal fission of the mitochondrion, while associated with the larger granule. Sokolska(8) has also depicted a similar process, but has applied a different interpretation to it.

It may be demonstrated, quite conclusively, that only the rod-shaped elements are of mitochondrial material, because intravital stains, which are specific for the latter, fail to show a similar selective action for the larger associated granules, and also because the associated granules gradually disappear during encystment and are totally absent in the mature spore (fig. 6). A study of the staining reaction, behavior, and morphology of these granules in mitochondrial preparations is important, because several authors who have depicted similar bodies suggest that they are of Golgi material.

In shape these cell bodies, which Horning(7) has termed vegetative granules, may be pyriform, spherical, or quite irregular. They may be detected under low powers of the microscope as large coarse granules distributed at random within the cytoplasm, and they display no migrational behavior in relation to the nucleus or the cell wall (fig. 2).

Bearing in mind the method of feeding of this astomatous ciliate and also the reaction of these curious granules to different techniques, the theory suggested by one of us(7) that these bodies, which have been suggested to be of a proteid nature, are food-storage products or vegetative granules seems strongly supported by their behavior during different phases of the metabolism of the organism and during encystment.

Apart from this, the aggregation or condensation of this lightly staining material at the surface of the mitochondrion is significant when correlated with the conception of the enzymatic nature of mitochondrial activity, which has received support recently by several authors(9, 10). The theory that the chondriosomes are loci of protein synthesis by virtue of the proteolytic enzymes which, it is suggested, are located

within them or at their surface, supports the contention that these associated granules are food-storage material which has been synthesized under the influence of the mitochondria.

Experiments on variations in the metabolism of *Opalina*, produced by isolating the organism in Ringer's solution of suitable hydrogen-ion concentration and starved, have shown that the vegetative granules disappear from the cytoplasm and at the same time the mitochondria undergo characteristic changes. Losing their association with the atrophying vegetative grains, the mitochondria increase in numbers by a process of transverse binary fission (fig. 3). It is certainly significant that, when under normal conditions synthesis of the vegetative material is apparently proceeding at the surface of the mitochondrion, the latter does not appear to undergo this process of fission.

Similarly, it has been shown that mitochondria in plant cells are fundamentally concerned in the formation of plastids, which are synthesized under their influence(11, 12, 13). Mitochondria within the plant cell apparently differentiate into plastids which are frequently referred to as transformed chondriosomes(14). However, our own observations, within the animal cell, demonstrate that the synthesized material, after attaining a certain size, becomes disassociated from the surface of the mitochondrion and migrates independently within the general protoplasm prior to absorption (fig. 2).

Intravital observations were made with the sodium salt of diethyl safranin monocarboxylic acid, prepared from Janus green by the hydrolysis of the nitrile, as well as with Janus green B. These studies again demonstrated the characteristic shape and orientation of the mitochondrial material, and at the same time failed to display a similar selective absorption for these stains in the case of the vegetative granules.

#### *b. Nyctotherus*

The mitochondria of this heterotrichan are relatively much smaller than those of *Opalina* and have been described in detail in a previous paper by Horning(15).

In addition to the above observations repeating previous work, it was noticed that surface sections of the cuticle stained with haematoxylin showed quite clearly the myonemata, which Horning suggests are responsible for the longitudinal polarity of the peripheral mitochondria in *Opalina* or the transverse orientation in the case of *Nyctotherus*. Associated with the myoneme threads in both organisms were extremely fine granules, scarcely exceeding the width of a cilium, placed in a series beneath the cilia and in close relation to the under surface of the cuticle (figs. 5, 7). These structures were obviously the basal granules described by so many morphologists in the ciliate Infusoria. The basal grains appeared in deeply stained haematoxylin sections and occasionally in silver preparations, and are exceedingly minute in comparison with other cell inclusions, owing to their position and close relation to each individual cilium.

#### STRUCTURES REVEALED WITH THE SILVER IMPREGNATIONS AND PROLONGED OSMICATION

Sections of *Opalina* treated with the Cajal and Da Fano techniques depict strongly differentiated, polymorphic bodies scattered irregularly in the cytoplasm. They show no characteristic orientation and in general morphology and appearance closely resemble the scattered Golgi apparatus frequently detected within the cytosome of metazoan germ cells, and are similar to those structures figured by Nath in the ova of *Culex* (16). They are distinctly coarse in texture and vary in size and shape from irregular granules to highly twisted, snake-like elements sometimes as long as the radius of the nucleus (fig. 4). Some of the contorted Golgi material may be aggregated in clusters within the central areas of the cytoplasm, while toward the periphery of the cell the granular form is more common and was observed to be merely the result of portions of the elongate Golgi elements being cut in transverse section. There can be little doubt that these structures, revealed by the silver techniques, which contrast so strongly both in morphology and behavior with the mitochondria, are Golgi material. In preparations where the cytoplasm is sufficiently homogeneous the mitochondria may

be detected as faint golden to dark brown rodlets in their characteristic position, and similarly the irregular vegetative grains have been recorded by photography as entirely distinct bodies from the Golgi elements (fig. 4).

#### THE GOLGI BODIES OF NYCTOTHERUS

The Golgi structures present in the cytoplasm of this holozoic infusorian consist of polymorphic bodies clearly differentiated from the general protoplasm and display no characteristic orientation within it. The Golgi substance varies from short rod-like bodies to twisted filaments and has a similar morphology and appearance to those already described in *Opalina* (fig. 7).

#### DISCUSSION

King and Gatenby(1), using Champy-osmium fixation, have described an aggregation of irregular, osmiophile bodies within the endoplasm of *Opalina ranarum*, which they presume to be Golgi elements. In addition, these pyriform bodies are shown to have a relationship with the cilia, suggesting a homology between the parabasal bodies of flagellates, which are held by Grassé(2) to be of a Golgi nature, and the pyriform osmiophile bodies of *Opalina*.

From a study of the figures depicted by King and Gatenby the number of these bodies, which are indeed relatively large cell inclusions, is seen to be vastly less than the number of cilia present on the sectioned cell wall. The enormous numbers of closely aggregated cilia on the surface of this holotrichous infusorian would make it seem unlikely that each cilium could be in separate connection with so large a structure. Further, these authors, in their enlarged drawing, show no evidence of the fine basal granules previously described by Minchin(17) and also shown by us to be sufficiently small to appear at the base of each individual cilium.

The evidence that the pyriform osmiophile granules of King and Gatenby are the vegetative grains previously described by Horning is further strengthened by the work of Sokolska and by King's subsequent criticisms of it(6). Using

a chrome-osmium technique, Sokolska(8) has recorded observations from preparations which appear to be similar to those recorded in this and preceding papers, although her interpretations are difficult to sustain. She depicts, quite clearly, the darkly staining mitochondria orientated longitudinally and each in association with a faintly staining granule, but contends that they are merely osmiophile loops and bands encircling a 'bound secretion,' and are therefore of Golgi material. Her observations also dealt with the typical bifurcation of the osmiophile band which Horning(7) interprets as longitudinal fission of the mitochondrion.

In reviewing the work of Sokolska, King(6) suggests that the term 'bound secretion' is inappropriate, as the pyriform granule is never observed actually within the interior of the chromophile band, but rather applied to its frequently concave surface, simulating, as King suggests, the chromophile and chromophobe portions of the Golgi apparatus of many metazoan cells. As Sokolska failed to show the supposed relationship of the chromophobe granules to the cilia, King does not support her previous suggestions that these granular structures are a secretory product of the osmiophile bands.

The evidence dealt with in earlier sections leaves little doubt that the longitudinally orientated, darkly staining elements are mitochondria, and the fact that both King and Sokolska have demonstrated either the vegetative granules or the mitochondria and have failed to reveal the characteristic elements which we hold to be the true Golgi material is perhaps merely confirmation of the opinion of most cytologists that Golgi techniques involving prolonged osmication may show mitochondria in place of the true structures.

Da Fano's cobalt-silver-nitrate process is criticized by some as a technique for invertebrate material, owing to the preliminary formalin fixation. In the case of *Opalina*, however, bad fixation may readily be detected by the extreme plasmolysis and disruption of the general protoplasm and nuclear membrane. With this technique it has been possible to demonstrate, in obviously well-fixed preparations, cell bodies

having the characteristics of the Golgi elements of the Sporozoa and other cells and, in addition, to show the independence of these bodies from mitochondria and vegetative granules which appear as golden brown rods and granules alongside the blackened Golgi material in the cytoplasm (fig. 4).

Hence, unlike previous investigators, we consider these three types of cell inclusions in *Opalina* as distinct, both by their morphology and their reactions to silver and osmium techniques and by their morphology and orientation within the cell, as a comparison of photomicrographs 1, 4, and 7 will show.

The similarity in appearance between the so-called Golgi elements described by King and Gatenby, the 'bound secretion' of Sokolska, and the granules which we have held to be synthesized by the mitochondria is too close to assume that they are separate cell bodies. In well-fed individuals the inner areas of the cytoplasm are so closely packed with vegetative grains and mitochondria (fig. 2) that it is difficult to conceive of a second series of equally large granules existing separately as so-called Golgi material.

The close relationship of the vegetative granules with the mitochondria, whose probable function is to build up protein at their surfaces, and the fact that these granules may be caused to disappear in starved *Opalina* or in individuals in a medium of extreme acidity or alkalinity make their supposed relationship to the cilia a matter for further confirmation, which in spite of repeated attempts we have failed to obtain.

In some sections blackened granules are found in *Opalina* and *Nyctotherus*, scattered in the ectoplasmic zone where the true rod-like Golgi elements seldom appear (fig. 4). These granules can have no relation to the cilia, because they are scattered too much at random to form the intracellular points of origin of the numerous and regularly disposed cilia. It is unlikely that they are of mitochondrial substance, because other techniques invariably show a segregation of the mitochondria in a zone of variable width, but never extending to the under surface of the cuticle. By studying serial sections



these coarse grains were shown to be merely transverse sections of the elongate Golgi material which is frequently aggregated in large masses immediately beneath the cell wall and therefore becomes frequently cut in section in all directions.

In cysts of *Opalina*, Golgi elements have not been detected, although mitochondrial preparations have been previously recorded by Horning(7) and also during this present investigation.

Round granular mitochondria are found to be aggregated around the periphery of the cysts—an orientation which this author holds to be a surface-tension effect(18) (fig. 6). Occasionally in slightly degenerate cysts, formed in Ringer's solution of an unsuitable hydrogen-ion concentration, the mitochondria were observed to enlarge, to stain less deeply, and to show a partial transformation into fat droplets(1). In suggesting the probable secretory function of the Golgi apparatus in Sporozoa, Joyet-Lavergne and King(4, 5) have demonstrated the presence of lipoid granules and fatty spheres in the gametocyte of *Adelina* and the spores of *Haplosporidium*. As these authors have not demonstrated the mitochondria as well as the Golgi apparatus in these organisms, it seems premature to contend that the fat droplets arise solely from the Golgi apparatus. So many investigators(19, 20) have shown the intracellular origin of fat to be the result of chondriolysis and one of the main evidences of cellular degeneration.

#### SUMMARY

1. Three types of cell inclusions are demonstrated within the general protoplasm of the binucleated *Protopalina*, parasites of *Hyla aurea*. These are considered to represent mitochondria together with associated, synthesized vegetative granules and Golgi bodies, as evidenced by their behavior, morphology, and staining reactions.

2. The Golgi material is shown to consist of irregularly twisted rods and granules scattered at random in the cytoplasm, but possessing a very distinct morphology and reac-

tion to different techniques when compared with the mitochondria.

3. No relationship could be detected between these vegetative structures and the cilia as has been previously described.

4. These observations have also been extended to a similar study of the cytoplasmic organs of *Nyctotherus cordiformis*, and it has been possible to demonstrate Golgi bodies of similar appearance within this organism as well as to show again the nature of the basal granules and their relationship to the cilia.

5. The procedure of identifying the mitochondrial elements with great care is recommended as a preliminary means of studying the Golgi apparatus of Protozoa, particularly where osmication techniques are used exclusively.

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### EXPLANATION OF PLATES

All illustrations are from untouched negatives of binucleate *Opalina* with the exception of figure 7, which is from *Nyctotherus*. The objective was a Zeiss 2-mm., apochromatic, and a condenser of the oil-immersion type.

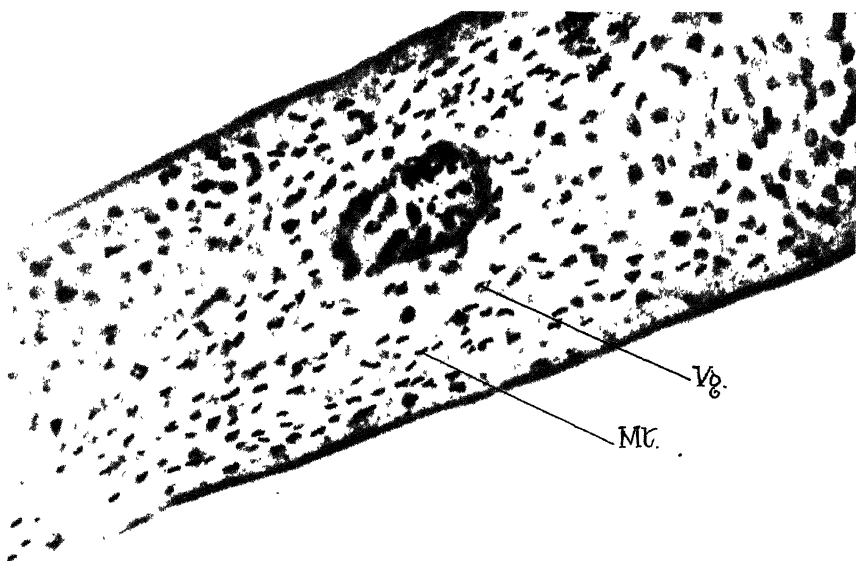
Figures 1, 2, 3, 5, and 6 were taken from preparations that were treated by the osmo-chromic method and stained with Heidenhain's iron haematoxylin and occasionally counterstained with eosin. Figure 4 was made from material previously treated by the Da Fano cobalt-silver-nitrate technique and figure 7 is a Cajal silver-nitrate preparation counterstained with neutral red. All sections were from 3 to 5  $\mu$  in thickness.

### · PLATE 1

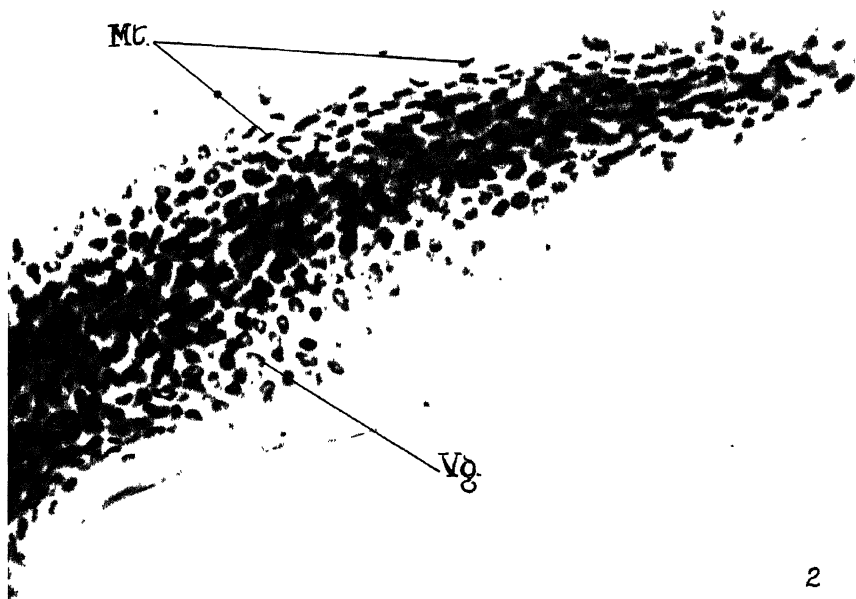
#### EXPLANATION OF FIGURES

1 Longitudinal section through *Opalina*, demonstrating darkly staining rod-like mitochondria, together with the lightly stained vegetative granules, which are shown either in close association with the mitochondria or lying independently within the general protoplasm. Observe curious longitudinal polarity of mitochondria to axis of cell. The nucleus is clearly depicted within center of field. *Mt.*, mitochondria; *Vg.*, vegetative granules.

2 The same, showing the contour of the vegetative granules in sharper focus than those depicted in figure 1. *Mt.*, mitochondria; *Vg.*, vegetative granules.



1

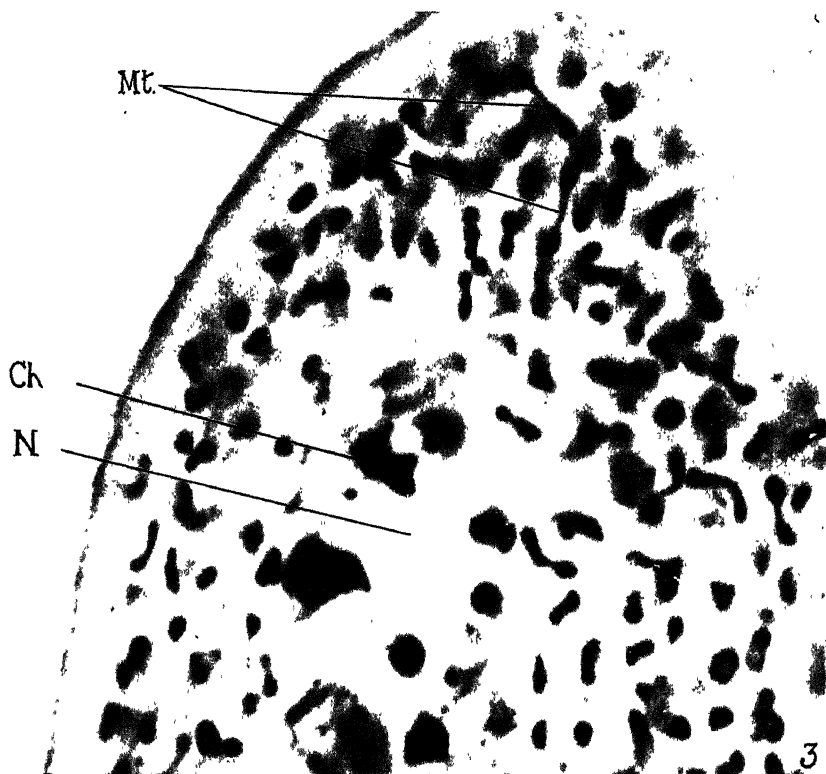


2

## PLATE 2

### EXPLANATION OF FIGURE

3 Depicting section through nuclear region of an *Opalina* which has been previously isolated and starved in a Ringer's solution of suitable pH, prior to fixation. Many mitochondria (*Mt.*) were fixed while undergoing transverse binary fission. Observe that mitochondria become disassociated from the vegetative grains while undergoing division. Cilia may be seen in periphery of the cell. *N.*, nucleus; *Ch.*, chromatin.

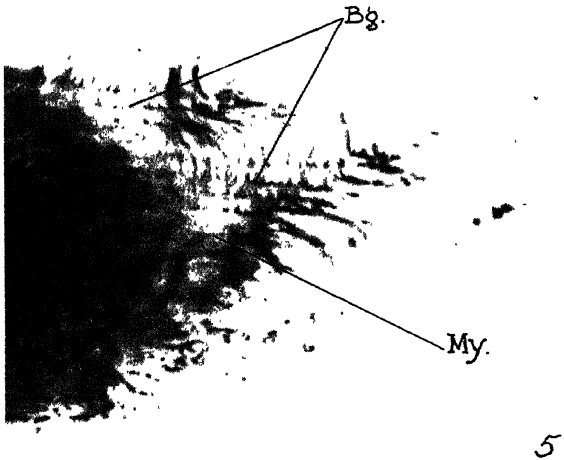
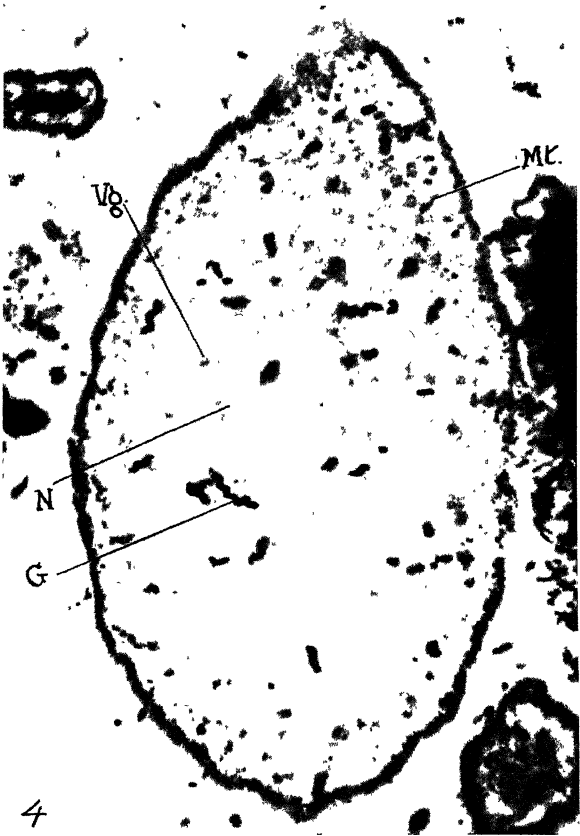


### PLATE 3

#### EXPLANATION OF FIGURES

4 Longitudinal section through nuclear region of *Opalina*, depicting polymorphic Golgi structures together with lightly staining vegetative granules which contrast with the darkly stained Golgi material. A rod-shaped, lightly stained mitochondrion, marked *Mt.*, is also revealed by this technique and in original preparation was seen as a golden brown structure. *N.*, nuclear membrane; *G.*, showing snake-like Golgi body near nucleus; *Vg.*, vegetative grains; *Mt.*, mitochondrion.

5 Showing darkly stained basal granules, which are attached to the base of each cilium. The myoneme threads which run parallel to and beneath the rows of cilia are also depicted. *Bg.*, basal granules; *My.*, myoneme threads.



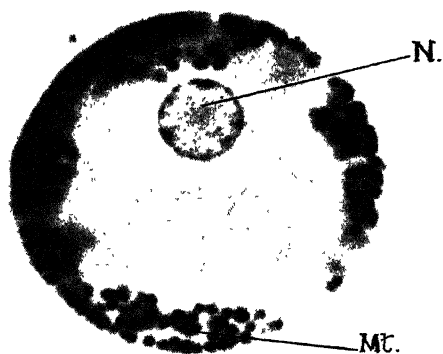


## PLATE 4

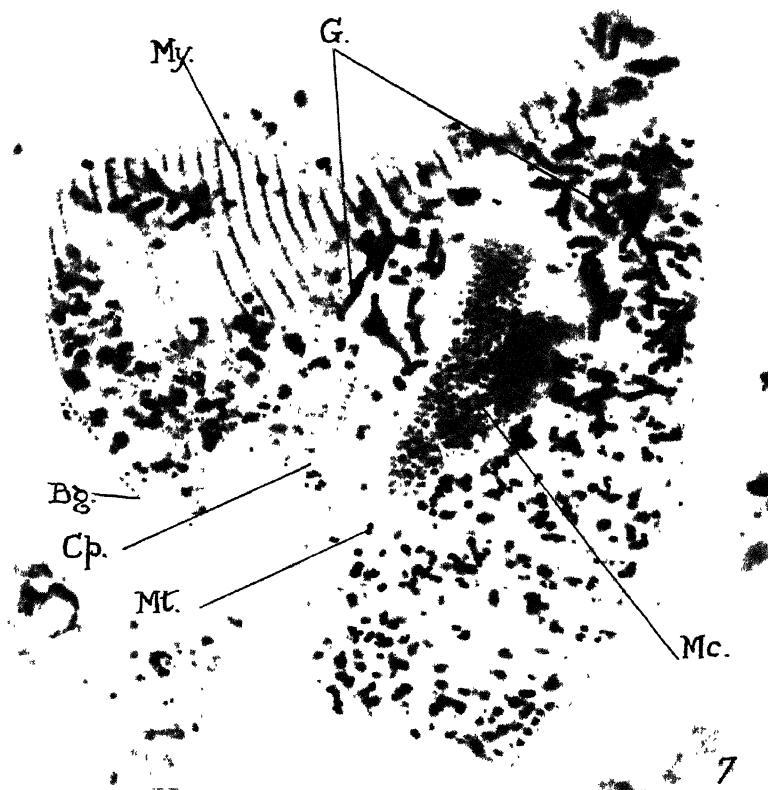
### EXPLANATION OF FIGURES

6 A section through an *Opalina* cyst, showing peripheral accumulations of mitochondria. *N.*, nucleus; *Mt.*, mitochondria.

7 Longitudinal oblique section through *Nyctotherus*, showing the bent, elongate Golgi structures within the superficial cytoplasm, as well as the small polymorphic Golgi bodies within the deeper portions of the cell which represent sections of portions of the Golgi material cut in varying planes. *G.*, Golgi material; *Mt.*, mitochondria; *Cp.*, cytopharynx; *Mc.*, macronucleus; *My.*, myonemata; *Bg.*, basal granules.



6



7



# THE EFFECTS OF VITAMIN-E DEFICIENCY ON THE DEVELOPMENT OF THE CHICK<sup>1</sup>

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ONE TEXT FIGURE AND FOUR PLATES (FOURTEEN FIGURES)

## AUTHOR'S ABSTRACT

In an investigation of the hatchability of eggs from hens fed on a diet deficient in vitamin E Card found that all embryos from these eggs died during development. Study of these chicks showed that a variety of conditions were responsible for death:

During early development the rate of growth and differentiation was definitely slower than under normal conditions, but malformations were rare. Some embryos died during the first two days, due to disintegration of the circulatory system or its failure to become established. At the end of the fourth day there was a definite critical period which few specimens survived and by this time distinct pathological conditions had arisen in extra-embryonic structures. These involved wiping out of the vitelline circulation by establishment of a lethal ring in the blastoderm. This structure was produced by intensive cell proliferation in the mesoderm which resulted in choking out vitelline blood vessels and their subsequent degeneration. It also caused obliteration of the exocoel, with consequent failure of the allantois to expand. In addition, many embryos showed profuse haemorrhage into the exocoel. The source of bleeding was most frequently in the atrium of the heart, and at the actual rupture peculiar cells occurred which were probably histiocytic mesenchyme cells.

Although death was due directly to causes enumerated above, the ultimate responsibility rests upon conditions set up by them, namely: starvation, asphyxiation, and loss of the medium (blood) by which to carry on metabolic exchange.

During the past few years, a series of experiments have been carried out by the Department of Animal Husbandry at the University of Illinois in an effort to find out something of the requirements of poultry for vitamin E. Among the problems investigated was a study of the effects of a lack of this vitamin on the hatching power of eggs. The results of these experiments are embodied in a number of reports by Card, Mitchell, and Hamilton, which have been published elsewhere (Card et al., '29, '30). When the general results of these investigations were available, it was seen, 1) that no appreciable improvement in egg laying or in hatching power was noticeable in a group of hens which had been given an extra quantity of vitamin E in the form of an extract, and, 2) that eggs from hens which had been altogether deprived of this vitamin for a sufficient length of time always failed

<sup>1</sup> Contribution no. 406 from the Zoölogical Laboratory, University of Illinois.

to hatch, even though development started if the eggs were fertile in the first place. Moreover, the hens used in the experiments continued to thrive and to lay in the usual manner.

Having in mind the extensive experimental work which has been done on the rat by Evans and his associates at the University of California and by various other investigators, the writer became interested in the possibility that an embryological study of chicks from these eggs during the early stages of incubation would throw more light on the question as to why death occurred in the course of development with such unfailing regularity. Accordingly, at a conference on the matter, it was arranged that the writer should collaborate with the original investigators named above in an effort to make the study more complete from an embryological standpoint. It is desired, therefore, to take this opportunity of expressing thanks to them for their courtesy in putting material at the disposal of the writer and it is hoped that the results set forth in this paper will justify the study.

In an embryological study of this nature, the chick offers certain definite advantages over the rat as the experimental animal. Among these are the following considerations:

1. The embryo of the chick develops in its own egg entirely apart from the mother. For this reason it is not subject to any such complex relation as that of the intimate placental connection between mother and foetus. Hence, there could be no masking of results by the mother-foetus relationship.

2. The chick has a definite store of nourishment in the form of yolk upon which to draw from the very first, and since this comes from a hen deficient in vitamin E, there can be no question concerning the lack of this nutritional element.

3. Another advantage lies in the ease of control of the incubation period and the fact that it is possible to candle the eggs as soon as the embryos begin to execute bodily movements. Hence dead individuals can be detected and may be removed from the egg before extensive disintegration has occurred. It is possible, therefore, to study individuals which

have died naturally, but in which histological conditions have not been much altered.

#### PRESENT STATUS OF THE PROBLEM

The discovery of the importance of vitamin E as a factor in the normal functioning of the reproductive powers of an animal and its rôle in embryological development was made by Evans and his associates at the University of California in 1922. Since then ('22-'27) they have carried out a series of interesting and valuable experiments on the effects of this substance, but their work on the rat still stands as the most thorough research in the field. Other workers, particularly in the fields of biochemistry and animal nutrition, have taken up the work and have investigated various angles of the problem. The pathological effects produced by lack of vitamin E have been investigated in the case of the guinea-pig, the mouse, and in the testis of the rat, but these studies have no bearing on the embryological aspects of the problem and hence will not be discussed. To date, however, no other studies have been made of the embryological and histological features surrounding the development of embryos under conditions involving lack of vitamin E.

In the case of poultry, feeding experiments involving E deficiency were carried out by Parkhurst ('24), but he was concerned only with the hatchability of the eggs produced. Card and his associates ('29, '30) have also made a similar series of investigations on the hatching powers of E-deficient eggs and on the effects of this nutritional deficiency on the egg production of such hens.

#### FEEDING CONDITIONS FOR THE ESTABLISHMENT OF VITAMIN-E DEFICIENCY

Before beginning a discussion of the problem in hand, it may be well to give a brief summary of the feeding conditions of the experiment by which the hens were deprived of vitamin E. This is fully reported in papers by Card and his associates which have been mentioned above, and the following is a condensed summary:

A flock of twenty-five Rhode Island Red pullets hatched March 28, 1929, were fed a normal ration until about eight weeks of age. They were then taken from the range on June 25, 1929, and confined to a house having a board floor and an adjoining wire-floored outdoor run. The hens were thus removed from all possible sources of vitamin E, and from that time on they were fed an all-mash ration which had been treated with ferric chloride in ether solution to remove any vitamin E which it might contain (Waddell and Steenbock, '28). The ingredients of the ration were:

Ground yellow corn,	60
Wheat bran,	15
Wheat middlings,	15
Meat scrap,	9
Salt,	1
Total,	<u>100</u>

To this mixture, 1 per cent cod-liver oil was added for the purpose of restoring the other vitamins, particularly A, which would also be removed by the method of treatment.

In the breeding season of 1930, seventeen of these pullets still survived, and beginning on February 1, 1930, they were mated to males of proved fertility. Beginning February 15th, the eggs were saved for incubation. The males were taken out of the pen at intervals and allowed to feed on untreated ground corn and wheat.

Incubation of these eggs gave the following results: A total of 317 eggs were incubated between February 26th and April 9th, but although forty-one developed beyond the ninth day, none hatched. Of the eggs collected between March 12th and April 9th, only nine developed beyond the sixth day, as determined by comparing the length of the embryos with normal ones.

Later, an attempt was made to remedy the nutritional deficiency by injecting wheat-germ oil and other substances containing vitamin E directly into the eggs, but these experiments produced no practical results, probably because the technique needed refinement. However, when the hens were

supplied with a quantity of wheat-germ oil daily, by mouth, there was an immediate improvement in the hatching power of their eggs. A final test to determine the hens' ability to store vitamin E was carried out by depriving the birds of wheat-germ oil once more, and this was followed by an immediate falling off in hatching results. From the summary which has been given above, it will be seen that we are dealing with a group of embryos, which, under the conditions of the experiment, are not able to complete development. The present investigation, therefore, is concerned with the embryological factors involved in causing death in these chicks.

#### METHODS OF STUDY

On April 16th, a setting of eggs from these hens, which had been reared on a dietary regimen deficient in vitamin E, was put at the disposal of the writer, together with a few available specimens from a previous hatching. All the eggs were put in the incubator at one time and arrangements made to remove a number at definite intervals of fourteen and ten hours, respectively (beginning at twenty-four hours), in order to obtain specimens as near the conventional laboratory stages as possible. Embryos were removed at the times stated as long as any of the eggs still contained living young, and at 110 and 120 hours all of the remaining eggs were candled, so as to make sure that dead specimens were not left in the incubator. After being taken from the incubator, the eggs were disposed of as rapidly as possible—the embryos being removed from the yolk and fixed in Bouin's solution with the least possible delay. In dealing with the earliest stages considerable difficulty was encountered because of the fact that delay in fixation was followed by rapid disintegration. This was overcome by a modification of technique which rendered the process of fixation very rapid.

After removal from the egg and prior to killing, the embryos were spread upon cover-slips, so that later they could be prepared for study either as total mounts or as serial sections. Specimens mounted in toto were stained with borax



carmine and the serial sections with iron alum-haematoxylin or Delafield's haematoxylin and eosin. The remainder of the material was simply preserved for reference or future preparation.

#### DETAILED OBSERVATIONS

For each of the stages mentioned above, except the twenty-four-hour stage, total mounts and serial sections were prepared. An attempt was made to use specimens which were most nearly representative of the conditions observed, but when extreme conditions occur, they are represented by both types of preparation whenever possible. At each of the stages studied, however, the anatomical details of development departed from expected conditions to a greater or lesser extent, and hence a brief summary of the outstanding features will be given below.

##### *Twenty-four-hour embryos*

*Body form.* These chicks had been in the incubator for twenty-six hours (two hours had been allowed for warming), but were not developed much beyond the condition usually seen at eighteen hours. Two had formed a very small head fold, but the remaining seven showed no advance beyond the primitive-streak stage.

##### *Thirty-eight-hour embryos*

*Body form.* The specimens removed at this time would compare in general appearance with normal embryos of twenty-eight to thirty hours' incubation. All four had a definite head fold.

*Ectodermal structures.* The brain was differentiated into the three primary vesicles and the neural folds were fused well back to about the level of the last somite.

*Endodermal structures.* A distinct foregut was apparent in the head fold.

*Mesodermal structures.* Nine somites were present. The vitelline veins were evident in front of the anterior intestinal

portal and these communicated with a simple tubular heart. Numerous blood islands were developed in the area vasculosa, but no extensive coalescence was apparent.

All four specimens seemed normal except for size and state of development.

### *Forty-eight-hour embryos*

*Body form.* Considerable variation was apparent in these embryos. Two of the five were abnormally small—suggesting the possibility that they had been dead for some time before their removal, but there was little evidence of disintegration. The remaining specimens approached more closely the typical forty-eight-hour condition, although not quite that far advanced. In these chicks both cranial and cervical flexures were apparent and a certain amount of torsion had been completed. The amnion covered the anterior half of the body.

*Ectodermal structures.* The brain in the smaller specimens showed the three primary divisions. In the larger embryos five divisions were present, but not clearly defined. These latter showed some development of the eye and ear as well. Cranial nerves V, VII, VIII, and IX were identified, but X had not yet developed.

*Endodermal structures.* Little change was noted in these structures other than the natural elongation of the foregut and the development of the pharyngeal pouches.

*Mesodermal structures.* In the smaller chicks there were eleven and twelve somites and the circulatory system resembled that described above for the thirty-eight-hour stage except that the looping of the heart was more pronounced. A noteworthy feature, however, was the fact that although the blood islands showed a slight amount of coalescence, they did not anastomose to form a series of channels communicating with the heart.

In the larger specimens nineteen, twenty-one, and twenty-four somites were marked off. Somatic blood vessels, both arteries and veins, had the characteristic arrangement, but aortic arches 1 and 2 only were present. It is to be noted that the third aortic arch has usually appeared by this time.

*Sixty-two-hour embryos*

*Body form.* Of the four embryos which were removed at this age, two were quite similar in appearance to the typical forty-eight-hour specimen, the third was smaller, but the fourth was entirely subnormal and showed practically no development.

*Ectodermal structures.* The brain showed five distinct divisions, and in the largest specimen the olfactory pit had begun to develop in addition to the eye and ear. Both epiphysis and hypophysis were also present. Of the cranial nerves, III, V, VII, VIII, and IX were developed, but X could not be found. It is to be recalled that nerve III ordinarily appears at sixty hours and nerve X is supposed to arise on the second day along with IX.

*Endodermal structures.* The largest of these embryos showed evidence of the beginning of the liver.

*Mesodermal structures.* The number of somites delimited varied considerably at this time, there being twenty-two, twenty-eight, and thirty. The circulatory system was much like that of the typical forty-eight-hour condition—the heart having a similar looping and aortic arches 1, 2, and 3 being present. In the largest embryo the fourth aortic arch was also beginning to appear.

In these embryos practically no evidence of abnormality was to be noted, unless a slightly greater thickness of the walls of the vitelline veins was an indication in the largest embryo. All three chicks were alive, with the heart beating rhythmically, when removed from the egg.

*Seventy-two-hour embryos*

*Body form.* Five embryos were obtained to represent this stage, and these again showed considerable variation in body form, ranging from specimens fairly similar to the forty-eight-hour type to others like the usual sixty-two-hour chick as far as body size and flexures were concerned. Very little evidence of limb buds or a developing allantois was apparent, although these structures are prominent in normal embryos

at this time. However, a definite tail fold was present in several of them.

*Ectodermal structures.* The brain and associated structures were somewhat more advanced in development, but otherwise the same ones were present. Cranial nerves III, V, VII, VIII, IX, X, and XI were found and there were also some slight indications of the beginnings of XII.

*Endodermal structures.* In the sectioned specimens of this age, the thyroid gland had begun to evaginate from the floor of the pharynx, although practically no trace of it could be found at sixty-two hours. The liver was well differentiated.

*Mesodermal structures.* In the smaller specimens the heart showed looping very similar to the forty-eight-hour condition, and aortic arches 1, 2, 3, and the beginnings of 4 were present. In the larger embryos the heart was more like the sixty- to seventy-two-hour types and aortic arch 1 had begun to dwindle. The cardinal veins and vitelline vessels were normal in arrangement.

In three of these embryos bleeding occurred into the exocoel as soon as they were removed from the yolk, in spite of the most careful handling. The fourth specimen was devoid of red blood except at the anterior and posterior margins of the blastoderm. In the area vasculosa the blood vessels seemed to have lost their integrity a short distance beyond the body of the embryo. In the sectioned specimens the source of haemorrhage was located, but this will be discussed more fully later. A sagittal section through an embryo at this particular stage is shown in the photograph, figure 1, and the apparently normal structure of the specimen is quite evident.

### *Eighty-six-hour embryos*

*Body form.* At this time four specimens were removed and all were alive. In general appearance they resembled embryos a little beyond the normal seventy-two-hour condition, with both limb buds and allantois beginning to show.

*Ectodermal structures.* These showed very little advance beyond a greater degree of differentiation and general enlargement of the brain. With regard to the cranial nerves, however, the olfactory I had appeared, in addition to those enumerated for the seventy-two-hour embryo.

*Endodermal structures.* Beyond greater differentiation and increase in size, these are also little different from the preceding stage; however, the allantois was present as a small diverticulum, about 1.2 mm. in diameter, from the hindgut. This is really about the size usually found in a seventy-two-hour specimen.

*Mesodermal structures.* The circulatory system within the body of the embryo appeared normal. The heart was very similar to the normal seventy-two-hour type and aortic arches 2, 3, 4, and 6 were present. Ordinarily, it will be recalled, arch 2 has disappeared by this time.

The evidences of abnormality at this time were very obscure. In two of the chicks nothing unusual could be noticed beyond the fact that there was marked retardation of development. One of the total mounts, however, showed a strong concentration of blood in the vessels inside the body. This particular specimen was mounted so as to include the entire blastoderm out to the sinus terminalis because of the fact mentioned above and because the sinus terminalis seemed to lack blood entirely. Study of the blastoderm revealed the beginning of disorganization of the blood system, which, taken in conjunction with the accumulation of blood in the body of the embryo, is of considerable significance in relation to the cause of death. This condition will be discussed in detail later. Of the serial sections of embryos of this stage, one showed extensive haemorrhage into the coelom and exocoel.

#### *Ninety-two- to ninety-six-hour embryos*

From the first setting of eggs three embryos were removed at ninety-six hours, but two of them were already dead. At 110 hours nearly all of the remaining chicks were also dead.

Hence when a subsequent setting of twelve eggs was made, six of the embryos were removed at ninety-two to ninety-three hours. Of these, four were alive and two had been dead for some time.

*Body form.* All the specimens with the exception of the dead embryos appeared normal in form, except that they were obviously retarded in development. In general, they resembled chicks of seventy-six to eighty hours' incubation, the head being well bent over, the allantois protruding prominently, and limb buds well formed.

*Ectodermal structures.* The brain and its associated structures showed a somewhat greater differentiation than the eighty-six-hour stage, but the prominent bulging of the brain which is usually so striking a feature in embryos of this stage was not particularly noticeable. The cranial nerves, however, had increased in number and I, III, V, VII, VIII, IX, X, XI, and XII were well defined. Ordinarily, VI is developed on the fourth day, but it could not be found.

*Endodermal structures.* These showed slightly greater differentiation and increase in size, but otherwise were not notably changed.

*Mesodermal structures.* The number of somites had reached the normal total with little exception. The mesonephros extended almost the full length of the body cavity, but bulged only very slightly into the coelom. Some evidence of the accumulation of primordial germ cells in the germ ridge was also found in certain of the specimens. As for the blood system, the heart presented some slight advance over the seventy-two-hour condition, aortic arches 2, 3, 4, and 6 were present, and the differentiation of pulmonary and systemic trunks was well advanced. The other vessels of the vitelline and cardinal systems were normal.

One of the dead specimens removed at this time presented a number of decided abnormalities, but the most remarkable feature about it was the presence of a dense ridge-like ring in the blastoderm entirely surrounding the embryo. Moreover, practically all extra-embryonic blood vessels in the

blastoderm were obliterated beyond the ring. In the living specimens removed at this time, the only unusual feature was the occurrence of haemorrhage from various blood vessels in some of the embryos.

### *110-hour embryos*

All the chicks removed at this time were dead, and consequently the remaining eggs were candled in order to ensure removal of any other dead specimens. Sixteen embryos were taken off, but eight eggs, about which there was some doubt, were left until the next regular period for removal. Of the specimens removed, eight had died some time previously and all these exhibited various degrees and types of malformation. Furthermore, nearly all of these embryos showed the well-marked ring on the blastoderm.

*Body form.* Since the best-developed specimens of this age appeared very similar to the ninety-six-hour E-free embryos, only three were sectioned and one mounted in toto. A general compacted appearance was noted about them, as if they had been confined in too small a space during development. The organs showed only a very slight advance over those present in the last stage.

*Ectodermal structures.* The brain exhibited a greater prominence than had been seen in any former specimens and cranial nerve VI had also appeared.

*Endodermal structures.* The digestive tube and the various structures which arise from it as diverticula, including the lungs, liver, and pancreas, seemed quite normal.

*Mesodermal structures.* The mesonephroi were well developed and the metanephric diverticula had appeared. In one specimen at least, a fairly prominent gonad was present. In regard to the circulatory system, the aortic arches were now reduced to 3, 4, and 6, while the pulmonary and systemic trunks were well established.

*120-hour embryos*

*Body form.* The eight remaining embryos were all dead at this time and all were removed from the eggs. In appearance they were considerably larger than the preceding group and were almost identical in shape with the normal ninety-six-hour type. The forebrain was bulged prominently and the allantois had increased to a diameter of about 4 mm. Some of the specimens showed evidence of haemorrhage and in every case the ridge-like ring on the blastoderm had either partially or completely interrupted the vitelline blood vessels. Two total mounts and one set of serial sections of this stage gave no other evidence of abnormality.

*Later stages*

Four other embryos were removed at later periods. These were all markedly underdeveloped, but in other respects appeared normal. Among them are six- and seven-day embryos which resemble five- and six-day normal types, respectively, and one which reached the comparatively old age of eighteen days. It would compare with the usual fourteen-day condition, but it was noted in this case that the yolk was practically all used up, in spite of the small size of the embryo.

## RATE OF GROWTH OF VITAMIN-E-DEFICIENT EMBRYOS

From the data given in the foregoing series of descriptions of the various stages studied, it is apparent that these embryos exhibit a marked retardation of development from the very inception of incubation. For purposes of comparison, the descriptions given by Lillie ('27) for the various stages have been considered standard. An attempt has been made to point out at each of the developmental stages prominent structures which are normally present at the stage described and which give some indication of the degree of development attained. However, although it was not always possible to present such data, there is definite proof from the details given that there is a progressive slowing up of differentiation and the rate of growth as the embryo gets older.



This has become so great by the end of the fifth day that the embryo then resembles, approximately, a four-day chick. This information has been summarized in table 1 and is illustrated by the graph, figure A.

#### CRITICAL PERIODS

During the first thirty-eight hours, while the embryo was assuming general body form and while the circulatory system was being established, some mortality occurred, but those embryos which survived this early period were usually able

TABLE 1

INCUBATION AGE	SOMITES		SIGNIFICANT STRUCTURES		DEVELOPMENTAL AGE <sup>1</sup>
	Normal	E-free	Present	Absent	
24 hours	6	0	Primitive streak		18 hours
38 hours	16	9	Heart simple tube		30 hours
48 hours	26	19-24		Thyroid	40-46 hours
62 hours	32	22-30	Aortic arches 1, 2, 3	Cranial nerve III	45-60 hours
72 hours	36	30-36	Cranial nerve III	Allantois	60-72 hours
86 hours	42	42	Aortic arch 2; allantois (72 hours)		76 hours
4 days	—	—	Cranial nerve VI		86 hours
5 days	—	—	Allantois (96 hours)		4 days
6 days	—	—	Body size		5 days
18 days	—	—	Intestinal loop		14 days

<sup>1</sup> It is desired to point out that in this table the developmental ages given are more or less arbitrary, since they represent an estimate from the limited number of specimens available. Therefore, on account of the great variability of the embryos, they will probably stand revision.

to keep on developing for the next few days. At the end of the fourth day, however, or very soon thereafter, there was a decidedly heavy mortality and very few embryos lived beyond this time. A few specimens reached an age greater than five days, but these belonged to a previous hatching in which all the embryos had been allowed to go on developing as long as possible.

It is evident, therefore, that the fourth day constitutes a definite critical period in the development of these vitamin-E-free embryos. This time is marked by the establishment of

certain unusual conditions in the embryo—particularly in the extra-embryonic structures—and these, in all probability, are responsible for the large number of deaths which occur.

Under normal conditions, as is well known, there is also a definite critical period in the development of chick embryos at the end of the fourth day (Jull, '30), but just what

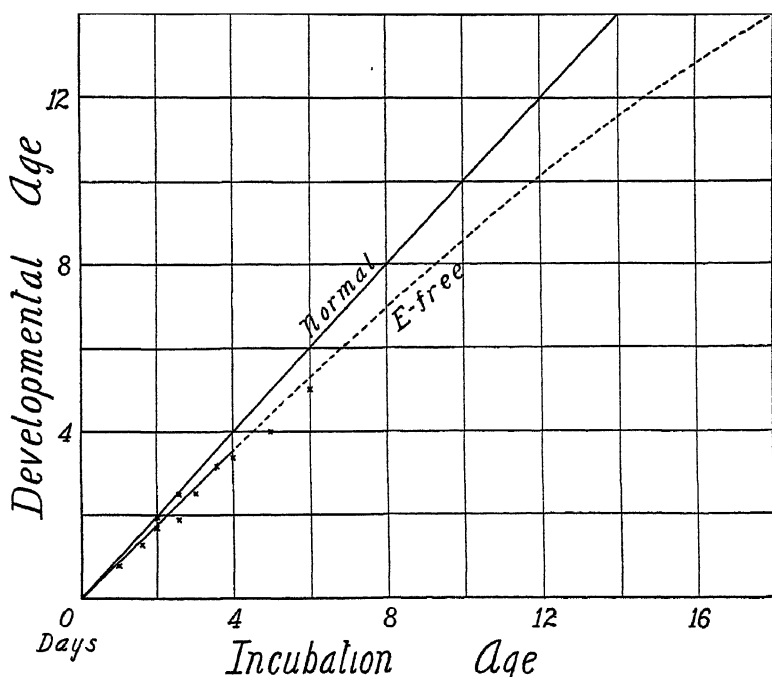


Fig. A Curves illustrating the rates of growth and differentiation of normal and vitamin-E-deficient chick embryos.

relation may exist between this fact and the conditions found in the present instance is problematical.

Due to the small number of eggs available, no attempt has been made to illustrate this condition graphically, but it may be observed that the conclusions reached regarding the period of greatest mortality agree well with the results tabulated by Card et al. ('30).

## CAUSES OF DEATH OF EMBRYOS

In order to determine the causes of the 100 per cent mortality which occurred among the embryos developing in these vitamin-E-free eggs, a thorough study of the various stages of development was carried out. The material used included both specimens which were living at the time of removal from the eggs and others which were already dead. The latter were studied in order to find out whether certain conditions observed in the living embryos were duplicated in those which had died naturally. This served as a check, therefore, as to whether or not these conditions were natural or were induced by the technical methods employed in killing and fixation of the chicks.

As the work progressed, certain observations were made regarding conditions in these embryos at the time of removal, and these furnished clues of considerable importance in finding the causes of death. These observations are listed below and they will be discussed separately in greater detail in the following pages.

1. Early disintegration of the blood vessels of the blastoderm appeared to have occurred in some of the embryos—particularly those which had died during the first few days.

2. Haemorrhage was very frequent among the older embryos.

3. A dense ridge-like ring developed around the blastoderm, so as to more or less completely encircle the embryo in many cases. This structure will henceforth be called the lethal ring.

4. In a few cases evident malformation existed.

Of the conditions enumerated above, by far the most common were the occurrence of haemorrhage and the development of the lethal ring on the blastoderm. Both of these often occurred in one individual, but sometimes only the ring was present.

## EARLY DISINTEGRATION OF THE BLOOD-VASCULAR SYSTEM

In a few of the earlier stages, the embryos had died either at the time that the circulatory system was being laid down or very soon after its establishment. In such cases, very little information could be obtained concerning the cause of death from a study of the embryo proper, but the blastoderm revealed conditions which were highly significant.

It was evident in some cases that the blood islands had failed to coalesce even at a comparatively late stage in development. In other cases of a similar nature, it was apparent that some development had occurred and the beginnings of definite blood channels had arisen in the blastoderm. These, however, had failed to anastomose to form the primitive circulatory system. A second condition was also manifested in which the blood vessels showed signs of secondary degeneration, although they had been originally well defined. This took the form of narrowings and interruptions of the blood channels, so that they presented a somewhat sketchy appearance. In a few cases accompanying this condition, there was also present a lethal ring around the embryo, but at some distance from it. Blood vessels could be seen within the ring, even though they were not altogether clear-cut, but beyond it there was often no indication of them.

No cause could be discovered for the failure of the circulatory system to become established and functional.

## HAEMORRHAGES

Among the older specimens which were dead when removed, a number were surrounded by a peculiar brownish fluid, and although this did not appear to be contained in the amniotic cavity, it was confined to a narrow area around the embryo. This region was bounded by the lethal ring. The brownish material was suspected of being exuded blood—a suspicion that was strengthened by the fact that a number of the living embryos between the ages of seventy-two and ninety-six hours bled more or less profusely almost immediately after removal from the yolk in spite of the most careful

handling. It was concluded, therefore, that these vitamin-E-deficient embryos were very susceptible to haemorrhage, particularly at later stages. Hence it was decided to section specimens of older embryos, both living and dead, in order to determine, if possible, the source of the bleeding and to find out whether the haemorrhages were accompanied by any unusual conditions which might be held responsible for their occurrence. Serial sections were prepared in transverse, frontal, and sagittal planes at thicknesses of 6 to 16  $\mu$ . These were stained with Heidenhain's or Delafield's haematoxylin and eosin.

Study of these sections indicated immediately that, so far as organogeny was concerned, the specimens were practically normal for the degree of development attained and no general changes in structure could be located which might be held responsible for the haemorrhages which had occurred. The study, therefore, narrowed down at once to a search for evidences of extravasation of blood, in order that the sites where this had taken place might be more carefully studied. Fourteen specimens ranging from 72 to 110 hours' incubation have been sectioned and studied for evidence of bleeding. Of these, eleven showed distinct haemorrhagic sites and in the majority of cases there was a considerable quantity of blood in the coelom or exocoele. Details of these findings are given below:

a. 72-hour, living, 2509B. This specimen showed considerable extravasation of blood into the coelom and exocoele. Two distinct haemorrhagic sites were located in the atrium of the heart. Both of these showed escaping blood cells as well as a mass of cells congregated about the rupture on the outside. Among this latter group of cells, small aggregates of cells could be identified which were of a type distinctly different from their neighbors (fig. 1).

b. 72-hour, living, 2507B. This specimen also showed distinct evidence of bleeding. The site of the haemorrhage was again found to be in the atrium of the heart and it was also marked by the presence of a group of cells outside the wall of the heart. Moreover, among these there occurred aggregates of small, distinctive cells of a type similar to those seen in the last specimen.

c. 72-hour, living, 2520B. This specimen showed a small amount of blood plasma in the coelom, but the source of this could not be located.

d. 86-hour, living, 2507C. In this embryo, extensive haemorrhage had occurred into the coelom and exocoel. A distinct rupture had occurred in the atrium of the heart and this was again marked by the presence of groups of small, distinctly different cells. In addition, other masses of these cells were congregated outside the heart in the coelom at various points where no break had occurred (fig. 2).

e. 86-hour, living, 2389D. This specimen appeared normal in every respect. No bleeding had occurred nor was it possible to locate any areas where it was suspected that bleeding might occur.

f. 96-hour, living, 2389F. This embryo appeared normal in every way and no bleeding had occurred at this time.

g. 96-hour, living, 2445H. In this chick a very strong haemorrhage had occurred and the source of extravasation of blood was again found to be in the atrial wall of the heart and in the allantoic artery. Cells were also congregated about the rupture in both places and clusters of the small peculiar cells were again identified.

h. 96-hour, living, 2509D. Haemorrhage had occurred into the exocoel, but only to a very limited extent. The source could not be located with absolute certainty.

i. 96-hour, living, 2521E. An extensive haemorrhage had taken place in this embryo. Its source was located in the atrium of the heart, where a distinct rupture of the wall was found. The cells congregated about the break also included the distinctive clusters as well as ordinary blood cells.

j. 96-hour, dead, 2494A. A slight amount of bleeding had occurred in this embryo, but its source could not be located with certainty. Probably, however, the escaped blood came from the smaller vessels of the liver.

k. 96-hour, dead, 2425D. Death had occurred in this embryo some time before removal. Extensive haemorrhage had occurred and several breaks were located in the atrial wall of the heart. These were marked by the usual type of cell.

l. 110-hour, dead, 2389E. Extensive haemorrhage had occurred in this specimen and several sites were identified. The most prominent was a rupture of the vitelline vein which opened into the coelom. In this region groups of escaped blood cells were clustered and among them were the usual groups of the small, differently staining cells. These latter were clustered about the edges of the opening. The heart, however, was packed full of blood corpuscles (fig. 3).

m. 110-hour, dead, 2509C. This embryo showed several sources of blood extravasation. One of these communicated directly with the

dorsal aorta, a second broke through the vitelline veins on the surface of the yolk sac, and the third opened into the blood sinusoids of the liver and thus formed a broad channel communicating with the right atrium of the heart and with the right common cardinal vein. Groups of the small heterotypic cells were identified at each site.

n. 110-hour, dead, 2445C. Blood had escaped from this chick in considerable quantity, but the heart and most of the blood vessels were gorged with blood. The source of the haemorrhages was in a most unusual place, namely, from one of the small sinusoids on the left side of the head near the eye. Degeneration was rather marked in this case, and the small cells usually associated with the rupture in definite groups were not so clearly defined and their identity was not so certain, although they were thought to be present.

From the details given in the foregoing records, it will be evident that haemorrhage occurred in a great variety of locations, but the most frequent site at which bleeding had occurred was the atrium of the heart. This is probably due in some measure to the fact that the wall of the atrium is particularly thin and hence would be most easily ruptured. However, it is also evident from the variety of sites located that the thickness of the wall of the ruptured vessel has little or nothing to do with the occurrence of a haemorrhage, for in some cases the break had taken place where the wall was comparatively thick. The actual sizes of the openings through which blood had escaped were usually very small. Thus, in the seventy-two-hour specimen (2509B), one rupture measured approximately  $45 \times 14 \mu$  and the other,  $35 \times 90 \mu$ . On the other hand, some of the breaks must have been as much as 1 mm. or more in width, judging from the number of sections through which they could be traced. The question arises, therefore, as to whether the occurrence of haemorrhages in these embryos is due purely to physical causes or to some physiological condition which has arisen.

In this connection, a careful examination of any of the haemorrhagic sites revealed conditions which are important. The region where bleeding occurred in any given case could usually be located by a more or less profuse extravasation of blood through a definite break in the wall of the confining

vessel (figs. 5, 6, 7, 8). In a few cases, the opening was partially plugged by a mass of coagulated blood plasma, but on the outer surface of the break there was nearly always one or several groups of cells arranged in a rosette-like cluster which could be readily detected and identified by certain definite characteristics. Sometimes these cells were also scattered, either singly or in groups, in other adjacent parts of the coelom. The occurrence of a group of these cells served as an almost unfailing diagnostic condition attending the loss of blood by an embryo.

*Cytological characteristics of cells associated with  
haemorrhage*

It has been pointed out that the characteristic manner of occurrence of these cells was in the form of a rosette-like cluster. This was particularly true when dealing with those located near the rupture of a blood vessel. The shapes of the individual cells account largely for their usual arrangement. The most frequent type encountered was a somewhat conical-shaped cell in which the small end was directed inward toward the center of the group in which it occurred. The shapes observed, however, were highly variable. This was true also of the nucleus, which was usually pear-shaped, although it was sometimes round, oval, lozenge, crescentic, elongate, or irregular. The nucleus was small and very compact, but the cytoplasm, on the other hand, was clear and quite homogeneous and showed no structural characteristics. A number of these cells are illustrated in figure 9 and a study of the photograph shows the clear cytoplasm contrasting strongly with the densely stained nucleus. Several of the various types of nuclei can be seen and comparison with adjacent blood corpuscles gives a good idea of the relative size and typical nature of these cells.

As for staining properties, these cells showed a marked affinity for basic nuclear stains, such as Heidenhain's or Delafield's haematoxylin, and with either of these dyes the nucleus was intensely colored. This reaction made the cells



easy to recognize, and for purposes of identification the former stain was most satisfactory.

In view of the frequent association of these peculiar cells with a haemorrhage, the question of their origin must be given some attention. It is admitted that there is scarcely enough material to settle the question absolutely, but two of the specimens offer conditions which appear to be significant in this respect. These are the seventy-two-hour embryo 2509B and the 110-hour specimen 2389E described above.

*Origin of the modified cells associated with haemorrhage*

In the younger specimen mentioned above, several more or less extensive haemorrhages had occurred, but the heart was still beating when the embryo was removed and there was still a considerable amount of blood in the vessels of the body. At the sites where extravasation had occurred, the usual rosette-like groups of cells were to be seen, but, in addition to these, there were many other clusters of them scattered about in the upper part of the coelom in the region of the heart and liver. Some of these were lying free in the body cavity, others appeared to be settled down on the surface of the neighboring tissues or to be actually embedded in them. A third condition was found in which the cluster of cells protruded out into the coelom on the end of a column or strand of mesodermal cells. In this specimen such columns were located in conjunction with the liver, heart, and left common cardinal vein. The appearance of a single group gave the impression that the mass of cells on the end of the column was intimately connected with the basal cells, but at the same time they were distinguished by the fact that they showed typical nuclei and were arranged in the characteristic rosette-like manner. Two such groups of cells and their supporting strands of mesoderm are shown in the photographs, figures 10 and 11.

In the older embryo a haemorrhagic site was identified in connection with the vitelline vein (figs. 3 and 7) and the typical groups of cells were also associated with it. However,

in this case, it was also possible to identify similar clusters of cells inside the vitelline vein, in the sinusoids of the liver, and in the atrium of the heart. Most of these groups were apparently free-floating among the blood cells, but at least two of them presented the appearance of having settled down or of being attached to the mesodermal lining of the blood vessels. No groups were found which were attached to and supported by columns of mesoderm cells.

Although not a great deal of material was available for a determination of the origin of these cells, nevertheless, because of the facts stated above, it is concluded that they arise from the terminal cells of the strands of mesenchyme projecting into the coelom. Hence they are to be regarded as a highly differentiated type of mesenchyme cell. As they increase in number, groups are probably detached or budded off and these become shifted to other near-by locations, where they settle down on the adjacent tissue. The migrations of the cell clusters could easily be accounted for by the movements of body fluids brought about by the pumping action of the heart or by the motions of the embryo itself. If the cells arise as suggested, they might occasionally get into the blood stream by proliferating into an open haemorrhagic rupture and by subsequently becoming freed from the parent mass which is located at the rim of the break in the wall of the blood vessel.

#### *Function of the cells associated with haemorrhage*

The very frequent association of these cells with a haemorrhagic site suggests the possibility that they may have some relation to this condition and that they may be responsible to some extent for the rupture. If this is the case, then they must have some sort of destructive action upon the walls of the blood vessels and upon any other tissues with which they might come into contact. In this connection, it will be recalled that in every case but one in which these cells were identified, they were found on the outside of the ruptured vessel, and not on the inside. In addition to the breaks in the walls of

the blood vessels, other regions have been found where the tissues for a considerable distance away from the actual rupture presented the appearance of cellular disintegration. This was true in some cases in which the embryo was still living. Furthermore, in such a case the modified mesenchyme cells could be found distributed all along the broken-down surface tissues. That such a condition could exist without its being related in some way to the occurrence of these cells hardly seems reasonable. Another peculiar condition encountered a number of times was the presence of a group of these cells among tissues that otherwise appeared normal. Whether or not this indicates an active invasion of healthy tissues remains to be more fully demonstrated. Finally, it was also observed in several cases, where a group of these cells had settled down on the wall of the heart, that a definite thinning of the tissues had occurred.

*Histological nature of the cells associated with haemorrhage*

The histological identity of the cells occurring at the site of the rupture of a blood vessel is fairly clear. If they develop, as has been suggested, by modification of the mesenchyme cells lining the body cavity, then their manner of origin recalls the mode of development of the various types of histiocytes and other wandering connective-tissue elements which are more or less phagocytic in function (Maximow and Bloom, '30, p. 133). This fits in well with the observed conditions attending the presence of these cells, and hence it may be that their primary function is to bring about the destruction of neighboring tissues, as has already been suggested.

In view of the indicated origin and function of these cells, they are probably related to macrophages and other cells of phagocytic action. It would appear, however, that their chief rôle is not to engulf and destroy foreign material, but rather to break down the walls of the blood vessels. For the present, however, it may be best to designate these cells simply as histiocytic mesenchyme cells.

## THE LETHAL RING

In the older embryos, particularly those which had reached at least the fourth day of incubation, there frequently occurred a peculiar ridge-like ring on the blastoderm. This structure has been called the lethal ring for a number of reasons which make the term decidedly appropriate, as will be apparent when it is discussed. The structure was encountered in at least twenty of the embryos which were old enough to show it. However, its presence was not discovered until an opportunity had occurred to make several total mounts. Hence, in a few of the embryos which might otherwise have shown it, not enough of the blastoderm had been preserved. After it was once discovered, as much of the blastoderm as possible was saved along with the embryo, and, if the specimen was not mounted immediately, it was all preserved for reference. Besides the total mounts which showed the ring, serial sections of portions of the blastoderm which included it were also prepared for study.

The earlier stages of these embryos, i.e., up to approximately seventy-two hours, showed no evidence of this structure in the blastoderm. In these the only indication of unusual conditions consisted of evidence of degeneration of the blood vessels in the area vasculosa. In the older specimens there were other peculiarities, in addition to the formation of the lethal ring, all of which were apparently related to its formation. The manner of development of this structure is well illustrated by three total mounts described below:

*Stage 1.* An eighty-four-hour embryo which was alive when removed was mounted in toto so as to include the entire blastoderm out to the sinus terminalis. In this specimen the vitelline arteries and veins had lost their capillary connections with the sinus terminalis at certain regions along the vessel. Moreover, careful study of these portions of the sinus terminalis revealed the presence of bulge-like prominences from the walls of the vessel which extended inward toward the embryo. At this stage these protuberances were very minute, but they are undoubtedly the first evidence of the conditions which eventually result in the formation of the lethal ring.

*Stage 2.* A second specimen of 110 hours' incubation, which had already died, revealed a further development of the ring. A streak-like thickening ran across the blastoderm on one side about midway in toward the embryo. The blood vessels extending out to this ridge from the embryo appeared normal, but beyond it they could not be traced as definite channels. Microscopic study of the thickened ridge showed peculiar bulgings which indicated that an intensive cell proliferation must have occurred. The whole blastoderm in this region had an abnormal appearance which can best be described as resembling a bubbling up of the tissues similar in appearance to that shown in figure 4.

*Stage 3.* The third specimen, illustrated in figure 4, is also a 110-hour embryo which was dead when removed from the egg. It showed the thickened ridge transformed into a complete ring encircling the embryo. The blood vessels extending to the ring appeared normal except for their thickness and the fact that some of them were turgid with blood, but, beyond the ring, no blood vessels could be recognized. It is also a notable fact that the vitelline veins returning blood to the embryo were all empty of blood except the few which anastomosed with branches of the arteries arising within the ring.

From conditions in the specimens described above, it is evident that the lethal ring first begins to develop near the outer margin of the blastoderm. This is accompanied by cell proliferation which involves the mesoderm (as will be shown later) and results in bridging the exocoele by strands of mesodermal tissue. The area of mitotic activity then seems to migrate inward toward the embryo, or the proliferating area may jump ahead, as it were, so as to involve a group of cells hitherto immune. That this is the case seems evident from conditions illustrated in figure 4 and also from the occurrence of just such an advance area in figure 13. By steadily advancing upon the embryo, the proliferating ridge eventually becomes a complete, encircling ring and thus the extra-embryonic vitelline circulation is wiped out. This cuts off the embryo from vascular communication with the yolk sac and results in restricting the cavity of the exocoele. Both of these conditions are significant when considering the causes of death.

*Histological structure of the lethal ring*

Pieces of the blastoderm from the region of the lethal ring were removed and sectioned for further study. The most satisfactory sections were cut transversely (i.e., radially with respect to the circular blastoderm). The detailed structures revealed by study of this material are described below.

Portions of the blastoderm from regions not quite as far out as the lethal ring, i.e., proximal to the embryo, exhibited the usual germ-layer formation—surface ectoderm, somatic and splanchnic mesoderm separated by the exocoel, and endoderm lying next the yolk. Moreover, the cells making up these layers were normal not only with regard to histological structure, but also as regards the number of cells in a layer.

Beginning at the ridge, however, conditions changed immediately (figs. 13, 14). The ectoderm consisted of a single layer of tall columnar cells, in contrast to the usual type of more flattened, squamous cells, and the whole sheet was thrown into numerous folds and ridges. Somatic mesoderm, which ordinarily consists of a single layer of cells beneath the ectoderm (or at most two or three cells), was made up of a reticular layer composed of many cells which filled up the cavities formed by the folds of the ectoderm. The splanchnic mesoderm was either in direct contact with the somatic layer or joined to it by bridging strands of cells which reached down from it. A condition somewhat similar to this has been noted by the writer in very early stages of chick embryos, but the occurrence is more or less of a rarity and is always very inconspicuous. In the region where the mesoderm layers were in contact there was frequently an intermediate layer of non-cellular material which had different staining properties. This is considered to be some kind of secretion. In this region, therefore, the exocoel was obliterated by the contact of the mesoderm layers and non-cellular material—a condition which explains why the blood of the embryo remained in a restricted area near the body of the embryo, hemmed in by the ring-like ridge, when a haemorrhage had occurred. The endodermal cells appeared normal

near the inner margin of the ring, but beneath the proliferated mesoderm they were frequently broken up and often completely disintegrated.

Since the splanchnic mesoderm is the layer through which the vitelline arteries and veins flow, it was found that these vessels were hemmed in and choked off by the proliferating mesoderm. Toward the inner border of the ring both arteries and veins can be readily identified, the former lying near the upper surface and being usually densely packed with corpuscles, while the latter are near the lower surface and are often almost devoid of blood cells. Deeper within the ring, where the mesodermal proliferation was more pronounced, the arteries could scarcely be distinguished from the surrounding closely packed tissue. Moreover, they were frequently invaded by strands of mesodermal cells. In this region, also, the dense crowding of tissue made the veins unrecognizable. Many variations of these conditions with regard to details and degree of development were found, but the description given is fairly representative of the average.

#### ABNORMALITIES

In about six cases, the embryos which were removed were slightly abnormal. Perhaps they should be more properly described as malformed individuals, but in any case they represent some departure from the normal form. Most of these specimens had developed to a stage approaching the seventy-two-hour type in which definite limb buds and a well-defined tail fold were present. In general, they gave the impression of having developed in a too closely confined space, with the various organs crowded together. They were also dwarfed to some extent, and one particular embryo was about half the usual length, even though it was just about as well developed as the others. Somewhat freakish development of certain parts was noted in a few cases, and in one specimen the brain with its prominent optic vesicles resembled the thirty-six-hour condition, although the rest of the body showed typical seventy-two-hour structures.

The presence of a lethal ring in all of these cases was the only other indication of any abnormality.

#### RELATION OF OBSERVED CONDITIONS TO THE DEATH OF THE EMBRYOS

From a study of the various conditions which have been found in the developing vitamin-E-free embryos, it is quite evident that a variety of causes is operating to bring about the death of the embryo in different cases. Moreover, since the actual body of the embryo undergoes more or less normal differentiation, the causes of death must, in general, be sought in the extra-embryonic structures and the conditions under which they develop.

During early development the rate of growth of the embryo evidently depends upon its ability to secure food. The responsibility of supplying this devolves upon the endoderm and the circulatory system. However, since there is a general retardation of the rate of growth long before the circulatory system becomes established, the lack of vitamin E must be responsible in some way for the inability of the endoderm to obtain food or for the inability of the embryo to use it after it is obtained.

When the vascular system becomes established, as it does, by thirty-eight hours in these specimens, the transfer of food to the embryo becomes a function of this system. Obviously, therefore, failure of the circulatory system to develop or a subsequent degeneration of this system must result in starvation and death of the embryo.

Should the embryo successfully pass through the period of establishment of the circulatory system, then normal but retarded development goes on until about the end of the fourth day. This is an indication of further inefficiency on the part of the blood system. By ninety-six hours, or soon thereafter, there has usually occurred one or several haemorrhages, any of which may prove fatal to the chick. Furthermore, by this time the lethal ring has usually become thoroughly developed in the blastoderm. The development of this



structure is accompanied by cell proliferation in the mesoderm of the yolk sac which effectively chokes off the circulation in the vitelline blood vessels. In this case, the death of the embryo must clearly be due to disturbances of the metabolic processes usually carried on by the extra-embryonic structures. The embryo is unable to obtain the proper amount of food, since a large part of the absorbing surface of the yolk sac is rendered non-functional. There must also be an accumulation of the waste products of metabolism, since the area of gaseous exchange is seriously restricted. Finally, the allantois is prevented from growing out to its full size by the fact that the exocoel has been hemmed in closely by the ring. Ordinarily, a more or less extensive development of the lethal ring is necessary before death ensues, but the mere interruption of the capillary connection of the vitelline vessels with the sinus terminalis has also been sufficient, apparently, to bring about this result in a few cases.

#### COMPARISON OF CONDITIONS IN THE RAT AND CHICK EMBRYO

Comparison of the conditions observed in the rat by Evans and his coworkers with those described for the chick shows that lack of vitamin E brings about the death of the embryo in practically the same manner in both cases. Although marked retardation of development occurred in both forms, it was observed from the very inception of development in the chick, whereas in the rat it was not noticeable for some time after development had started. In both cases, also, there was a marked proliferation of mesoderm. In the rat this occurred in the placenta, while in the chick it took place in the yolk sac. In the chick, however, it has been possible to demonstrate clearly that this condition brings about a choking off of the blood vessels of the yolk sac with the consequent production of starvation and toxic conditions which are ultimately responsible for the death of the embryo.

The occurrence of haemorrhages in the chick has been clearly demonstrated and it has also been shown that the region of haemorrhage is marked characteristically by the

presence of typical cells which have been identified as histiocytic mesenchyme cells. In the case of the rat, extravasation of blood into the placental spaces was found to have taken place and it was noted that this condition was frequently accompanied by the occurrence of cells with pycnotic nuclei inside the blood vessels of the maternal portion of the placenta. The escaped blood was considered to be maternal blood, but there was often a marked reduction in the number of blood cells in the vessels of the embryo. In the case of the chick, smaller blood cells with small nuclei have been seen in some specimens, but these are regarded merely as cell types which have already become differentiated.

As to the impairment of the haemopoietic function of the liver and blood islands in the chick because of the lack of vitamin E, there does not seem to be much evidence in favor of the supposition, although the condition was found to occur in the rat. On the contrary, there is every indication that the blood cells are multiplying rapidly—even where they are intensely packed in by the lethal ring—and are just as numerous as in the normal embryo. Furthermore, the rapid increase in the number of blood cells in the early chick embryo can scarcely be attributed to the activity of the liver, for this organ is just becoming nicely established itself. More probably, the normal increase is due to rapid mitosis of the primitive haemoblasts. It is the writer's opinion, therefore, that in the chick, at any rate, empty blood vessels signify that extensive haemorrhage has taken place with consequent draining of the blood vessels. In support of this contention ample evidence has been presented showing the frequent occurrence of haemorrhage not only in the vitamin-E-deficient group as a whole, but also in individual specimens.

#### SUMMARY

Under conditions involving a lack of vitamin E, it was found that chick embryos always failed to complete development—usually dying by the end of the fourth day or shortly thereafter. It was desired, therefore, to find out what

occurred in the developing chick to bring about such inevitable mortality. The facts disclosed by this investigation are as follows:

1. Developing embryos usually show practically normal differentiation as far as general structures and organs are concerned.

2. Retardation of the rate of development is noticeable within the first twenty-four hours of incubation and increases progressively as the period of hatching advances.

3. In a few cases the blood-vascular system may fail to complete development or may degenerate very soon after it is laid down. This brings about the death of the embryos in which it occurs.

4. A definite critical period in the development of these embryos occurs on the fourth day or thereabouts. The causes of death at this time are varied.

5. Embryos which have passed the early critical stages of development are frequently subject to haemorrhage, which may occur in a variety of places. One or several haemorrhages may take place in a single embryo.

6. Haemorrhagic sites are usually marked by the occurrence of groups of cells of a distinctive type which are considered to be histiocytic mesenchyme cells and which are thought to be involved in the production of the haemorrhage. The origin of these cells is probably from modified mesenchyme cells of the lining of the coelom.

7. A lethal ring is developed in the blastoderm as the embryo gets older. This structure entirely surrounds the embryo in its most complete form and involves intensive cell proliferation in the mesoderm of the blastoderm. In the regions where this structure is developed the endoderm is often completely disintegrated.

8. In embryos in which a lethal ring has developed, the circulatory system is choked off either by the interruption of the capillary anastomoses between the vitelline arteries and veins or by the extensive cell proliferation which takes place in the lethal ring.

9. The development of the lethal ring is frequently accompanied by modification of the histological types of cells which normally make up the germ layers of the blastoderm.

10. The advance of the lethal ring upon the embryo results in a restriction of the exocoel and this is attended by failure of the allantois to enlarge.

#### CONCLUSIONS

Eggs from hens which have been deprived of vitamin E in their diet are also lacking in this vitamin and hence embryos developing in these eggs die before the incubation period is well under way. Ordinarily, there is no marked abnormality in the body of the embryo other than a definite slowing up of the rate of growth and the occasional production of dwarf individuals. However, accompanying the death of the embryo characteristic pathological conditions are established which are in a large measure responsible for the death of the embryo:

1. In some cases degeneration of the circulatory system occurs, although no cause could be found for this condition.

2. Haemorrhages frequently take place in vitamin-E-free embryos after the third day and these result in draining of the embryonic blood vessels. In the chick, therefore, failure of the haemopoietic function of the liver would seem to be definitely precluded as an explanation of lack of blood in the embryo. The occurrence of haemorrhage may take place in a variety of sites, but these regions are marked characteristically by the presence of groups of histiocytic mesenchyme cells which have definite structural features by which they may be identified. These cells, moreover, are probably responsible for the occurrence of bleeding.

3. A lethal ring is developed in the blastoderm and it has generally surrounded the embryo completely by the end of the fourth or fifth day. This structure is formed by extensive cell proliferation in the mesoderm and is attended by histological changes in the ectoderm and mesoderm as well as frequent degeneration of the endoderm. As a result of the for-

mation of the ring the blood vessels of the blastoderm are completely choked off and ultimately disintegrate.

Death of the embryo always occurs and it is the direct result of the conditions enumerated above. The ultimate causes of death, however, are to be found in the effects produced by these conditions, namely: 1) Loss of blood due to haemorrhage as a result of which the means of carrying on metabolic exchange is no longer available. 2) Starvation, asphyxiation, and the establishment of general toxic conditions due either to haemorrhage or to the stagnation of the circulation consequent upon the development of the lethal ring.

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<sup>2</sup> The papers listed above are only those which have a direct bearing on the problem from an anatomical standpoint. Numerous other articles dealing with the biochemical, physiological, and pathological aspects of the subject are available and have been consulted by the author, but, for an extensive bibliography covering the entire field, the reader is referred to Sherman and Smith's recent second edition of "The Vitamins," which is listed above.

# PLATES

## ABBREVIATIONS

<i>a</i> , 4, 3, 2, etc., aortic arches 4, 3, 2, etc.	<i>i.c.</i> , internal carotid artery
<i>a.c.</i> , anterior cardinal vein	<i>l.</i> , liver
<i>all.</i> , allantois	<i>l.b.</i> , limb bud
<i>am.</i> , amnion	<i>m.</i> , mesonephros
<i>a.p.m.</i> , advance area of proliferating mesoderm	<i>m.c.</i> , supporting column of mesoderm cells
<i>at.</i> , atrium	<i>n.</i> , notochord
<i>bl.</i> , blood cells	<i>n.c.m.</i> , non-cellular layer in mesoderm
<i>b.</i> , brain	<i>o.</i> , oesophagus
<i>c.</i> , histiocytic mesenchyme cells associated with haemorrhage	<i>o.v.</i> , otic vesicle
<i>cl.</i> , cloaca	<i>p.</i> , pharynx
<i>d.</i> , duodenum	<i>p.c.</i> , postcardinal vein
<i>d.a.</i> , dorsal aorta	<i>r.</i> , rupture of blood vessel
<i>ect.</i> , ectoderm	<i>s.</i> , stomach
<i>end.</i> , endoderm	<i>s.c.</i> , spinal cord
<i>ex.c.</i> , external carotid artery	<i>som.</i> , somatic mesoderm
<i>g.</i> , gut	<i>spl.</i> , splanchnic mesoderm
<i>h.</i> , heart	<i>v.a.</i> , vitelline artery
<i>H.S.</i> , site of haemorrhage	<i>v.v.</i> , vitelline vein
	Roman numerals indicate cranial nerves

## PLATE 1

### EXPLANATION OF FIGURES

- 1 Sagittal section of a seventy-two-hour chick embryo.  $\times 13$ . Normal but retarded development is indicated. Note the ruptured wall of the heart.
- 2 Sagittal section of an eighty-six-hour chick.  $\times 13$ . The atrium of the heart is ruptured and much blood has escaped into the exocoel.

VITAMINE DEFICIENCY IN THE CHICK

F. B. ADAMSTONE

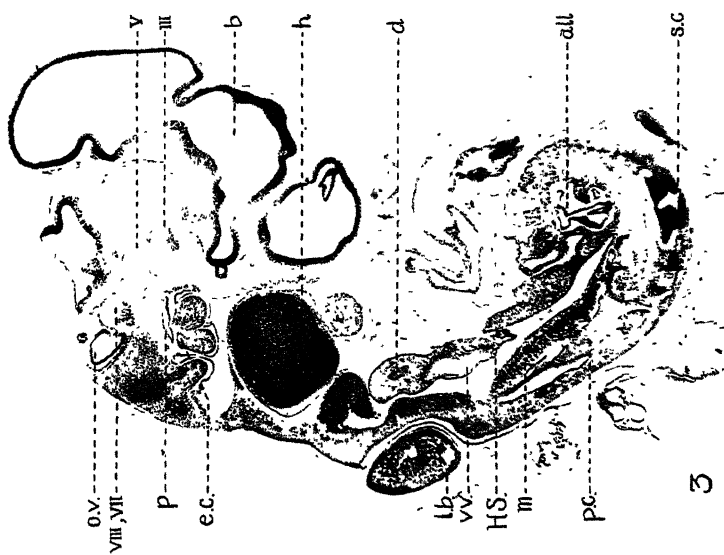




## PLATE 2

### EXPLANATION OF FIGURES

- 3 Sagittal section of a 110-hour chick.  $\times 13$ . The rupture is in the vitelline vein.
- 4 Total mount of a 110-hour embryo.  $\times 13$ . This specimen shows the lethal ring fully developed as well as disintegration of blood vessels beyond it.



3

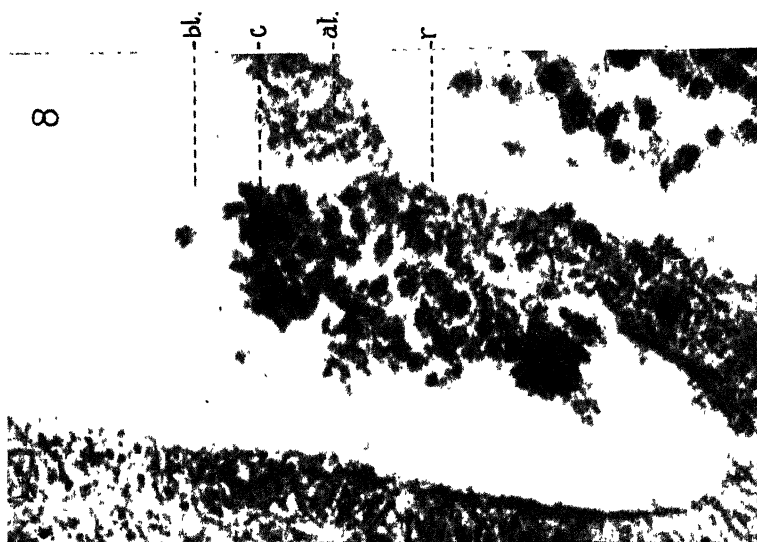
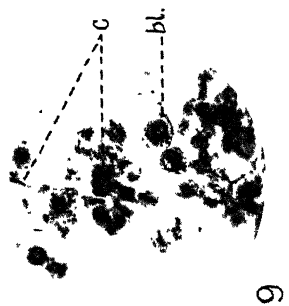
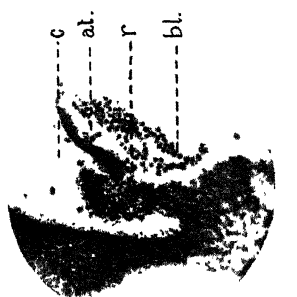
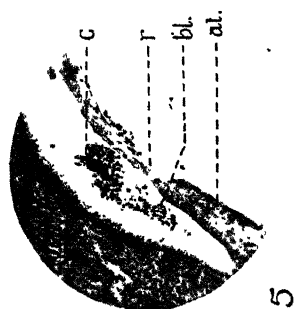


4

## PLATE 3

### EXPLANATION OF FIGURES

- 5 A portion of the wall of the atrium of a seventy-two-hour chick.  $\times 50$ .  
The rupture and escaped cells are clearly shown.
- 6 A second rupture in the heart wall of the same seventy-two-hour chick and separated from that shown in figure 5 by approximately  $300\ \mu$ .  $\times 50$ .
- 7 The ruptured vitelline vein shown in figure 2.  $\times 50$ .
- 8 Region of haemorrhage highly magnified to show rosette-like clusters of histiocyte cells among the escaped blood. They are easily recognized by their darker color.  $\times 140$ .
- 9 A single cluster of the histiocyte mesenchyme cells usually associated with a haemorrhage. The clear cytoplasm of these cells is evident as well as the denseness and variety of their nuclei.  $\times 240$ .



## PLATE 4

### EXPLANATION OF FIGURES

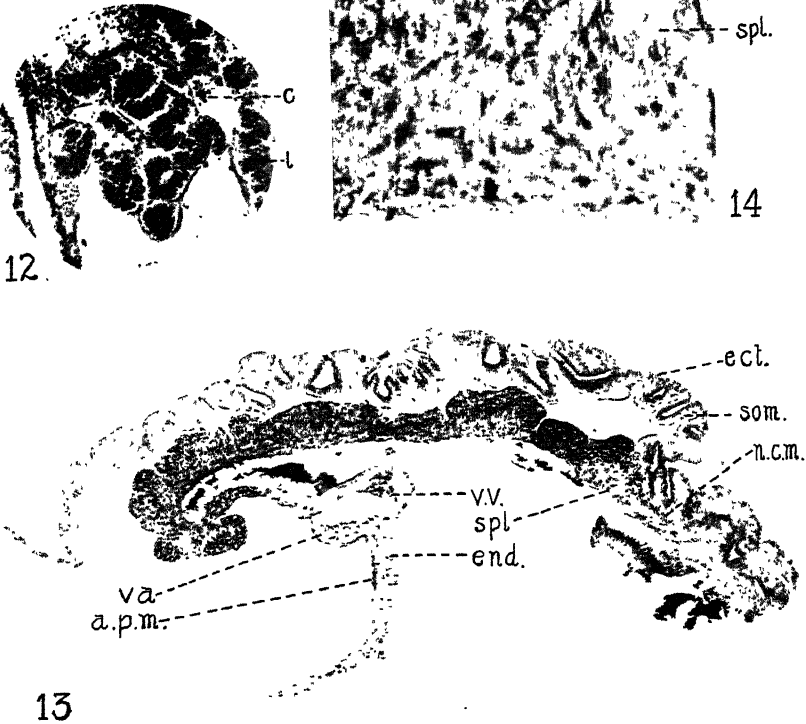
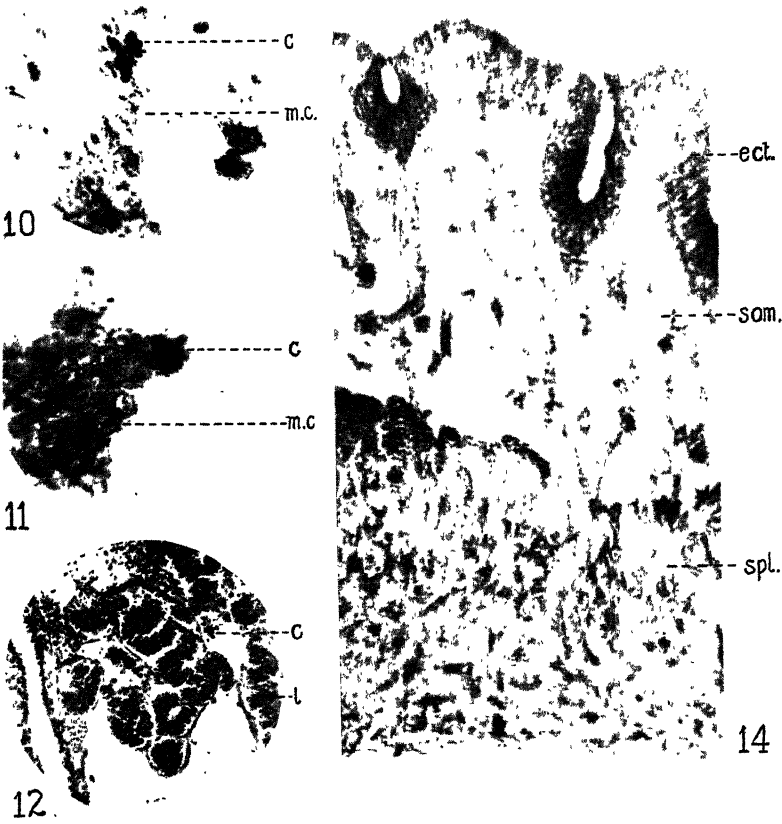
10 A group of the histiocytic mesenchyme cells from a seventy-two-hour embryo.  $\times 240$ . The supporting columnn of mesoderm can be seen.

11 Histiocytic mesenchyme cells similar to those in figure 10.  $\times 240$ .

12 A portion of the liver of a 110-hour embryo in which two groups of the histiocytic mesenchyme cells can be seen.  $\times 50$ .

13 Radial section of a portion of the blastoderm through the lethal ring of a ninety-six-hour embryo.  $\times 35$ . The modifications in the germ layers are clearly evident.

14 Small portion of the blastoderm showing histological details highly magnified.  $\times 270$ .





# A STUDY OF LIVING SPERM CELLS OF CERTAIN GRASSHOPPERS BY MEANS OF THE ULTRAVIOLET MICROSCOPE

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NINE TEXT FIGURES AND FOUR PLATES (TWENTY-FIVE FIGURES)

## AUTHORS' ABSTRACT

This paper discusses very briefly the ultraviolet microscope and the developments which have led to a successful technique for optically sectioning living cells, also the ultraviolet photomicrographs of living sperm cells of certain grasshoppers, which show clearly the spinning out of the chromonema from solid blocks of the diatene stage, the pairing of same in the leptotene stage, the development of the tetrads, the final distribution of the chromosomes in the resulting spermatids and their return to a spiral chromonema, each inclosed in its own vesicle. The details of the cytoplasm are equally well brought out.

## FOREWORD

This paper is the outcome of a pooling of interests of two investigators working ordinarily in quite unrelated branches of science, but with a common bond. There had been developed by one of us, a metallographer and microscopist, a very powerful tool which increases enormously the power of vision. The application of this new tool to metallurgical problems indicated that it should be of great aid in the biological sciences because of certain inherent characteristics of the optical system which were discovered. These matters have been discussed in a paper presented elsewhere ('30). They may be summarized briefly as follows:

About thirty years ago, the ultraviolet microscope was developed for biological research. At that time metallography was in its infancy. For many years ultraviolet microscopes have lain unused, collecting dust in out-of-the-way corners of biological laboratories here and there throughout the world—a scientific curiosity, seemingly of no practical use.



In learning how to use the equipment as a metallurgical microscope, certain inherent possibilities of the ultraviolet system were discovered. They were discoveries which since have made it possible to photograph the internal architecture of living cells; figuratively speaking, to place the microscope within a single living cell, itself microscopic in size, and to take photographs at very high magnifications of the structure within the cell—to photograph upward or downward on different planes spaced 0.00001 inch apart. In some cases as many as twenty or thirty photographs may be taken on as many different optical planes within the cell and generally without interfering, so far as one is able to detect, with the normal living functions of the cell. These methods bring within photographic range the structure of living things in a manner never before possible.

The ultraviolet microscope possesses several advantages over the visible-light microscope employing the best apochromatic objectives. Perhaps its best known advantage lies in the fact that by virtue of the shorter-wave-length light employed, it has about double the resolving ability. This one attribute alone provides man with a very powerful tool. The use of ultraviolet light instead of visible light enables one to work with living material free from all fixatives and stains and thus to approximate closely the normal living conditions of the particular material under study. For many years it has been known that organic material exhibits the phenomena of selective absorption toward ultraviolet light. By virtue of this selective characteristic, an unstained living cell which would appear practically structureless under a visible-light microscope appears much as though it had been stained when photographed by the ultraviolet microscope. The ultraviolet microscope selects a focal plane which is practically a geometrical plane, i.e., detail above or below the exact focal plane does not interfere with the image. It is this characteristic of the system which led us to the development of optical sectioning and which has enabled us to work at much higher magnification than is possible with the visible-light microscope.

The advantages of the ultraviolet microscope over the visible-light system may be briefly summarized as follows:

1. The resolving power is doubled.
2. The magnifying ability is trebled or quadrupled.
3. An exact focal plane may be selected (i.e., we may optically section a specimen).
4. Dyes, stains, fixatives, and all embedding operations are eliminated.
5. The material may be photographed in a condition which approximates closely its normal living condition.

The disadvantages of the ultraviolet microscope are perhaps twofold. All material does not transmit ultraviolet light. Material which is opaque to ultraviolet light, of course, cannot be studied by this method. Perhaps the greatest fault to be found with the ultraviolet microscope is its reaction on the operator. The concentration and the accuracy required for successful operation, the strain on the eyesight of the individual, and the nervous reaction prove tiring to the point of exhaustion at times. One can hardly realize how this could be so, but I seldom work more often with the ultraviolet microscope than part of every other day. If one is to achieve outstanding results, everything must be tuned to a high state of perfection—not only the apparatus, but the individual as well.

The technique of ultraviolet microscopy is far enough advanced so that it is now practicable to take one or two hundred plates in the course of a day's work. The number of unsatisfactory plates for all reasons probably does not exceed 2 or 3 per cent. Improvements in technique are resulting in a general upward trend in the order of resolution achieved.

In cooperation with Doctor Stark, the ultraviolet microscope has been applied to several biological problems. It is hoped that some of the preliminary results presented in this paper and discussed by Doctor Stark will prove of interest.

FRANCIS F. LUCAS.

The study of the development of the sperm cells of certain grasshoppers is dealt with in this paper, and primarily the mechanism of mitosis, concerning which the ultraviolet microscope has cleared up a number of debatable points.

It was just chance that *Melanoplus femur rubrum* was used, since a grasshopper of that species was the first to jump into the collecting net. Photographs of its germ cells gave us nearly a complete history of spermatogenesis.

A follicle from the dissected gonad was placed in a drop of Ringer's solution on a quartz slide, where it was cut in two about one-third of the distance from the distal end. Slight pressure was exerted upon the cover-slip placed upon this follicle. To prevent evaporation of the Ringer's solution, the cover-slip was fixed to the slide with vaseline.

The first photograph, figure 1, at a magnification of 500, presents a picture of several stages in one field. The largest cells are primary spermatocytes; the smaller ones, spermatids and their early transformation stages into spermatozoa, while the thread-like structures are nearly mature spermatozoa, some of them moving about in the solution before and after the exposure.

When the spermatogonia are ready for further development, the individual chromosomes are lost to view for a short time, to suddenly appear again, the mates of each pair side by side, as seen in figures 10, 11, 12, 13, and 14. These are all typical diatene stages as described by McClung ('27). Figure 12 is a photograph of *Rhomaleum* germ cells, cell *B* having a polar view of the X-chromosome; *C*, a lateral view of the X-chromosome with a distinct spiral thread as described by McClung ('99, '00). This spiral thread of the X-chromosome has been traced to the metaphase stage by Wenrich ('16) and others, proving the genetic continuity of this chromosome. This photograph also indicates clearly that the X-chromosome retains its separate vesicle, giving the appearance of a small independent nucleus lying next to the large one. The vacuolated form of the X-chromosome vesicle is present in figure 2 and in cell *D* of figure 13.

The characteristic processes extending out from the chromosomes, as if an intensive metabolic reaction were taking place between the chromatin and the nucleoplasm preceding a series of changes in the nuclear system, are evident in figures

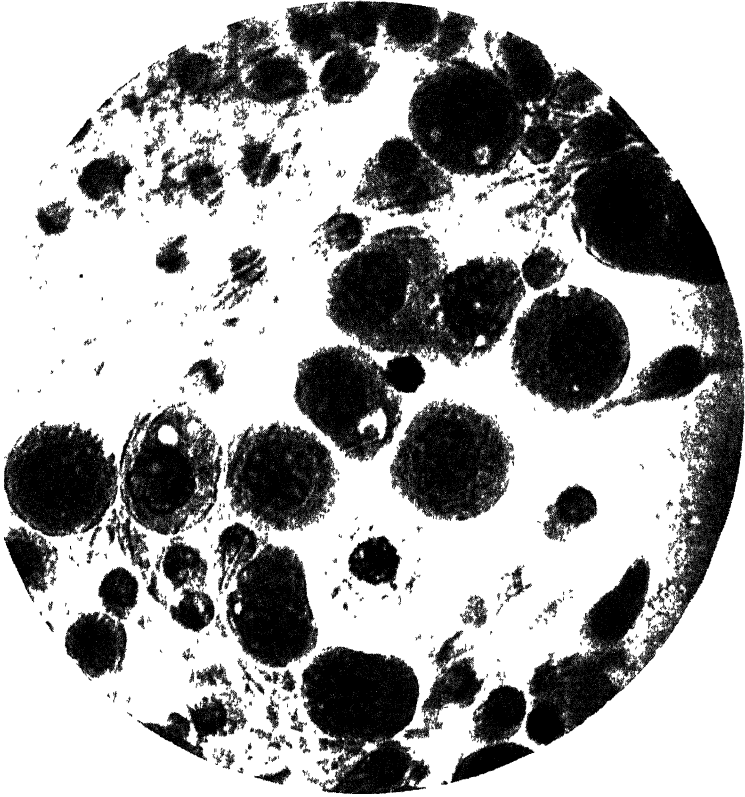


Fig. 1 Sperm cells of *Melanoplus femur rubrum*, magnified 500 diameters. Primary spermatocytes and spermatids in all stages of development. Mitochondria as granules in spermatocytes.

2 and 32. After this reaction, the paired diatene chromosomes become drawn out into very much convoluted threads which unravel into fine double threads, polar views of which are present in figure 16. This presence of the spireme thread had been noticed as early as 1882 by both Flemming and Strasburger in their studies of living cells during mitosis.

That this thread is the spinning out of the massive chromatin blocks (figs. 10, 11, 12, and 13) of the diatene stage has been described by Janssens ('01) in the spermatogonial prophases of Triton, by Davis ('08) and many observers in the preleptotene nuclei of Orthoptera, by Wilson ('12) in those of Hemiptera, and by Nonidez ('10) in those of Coleoptera.

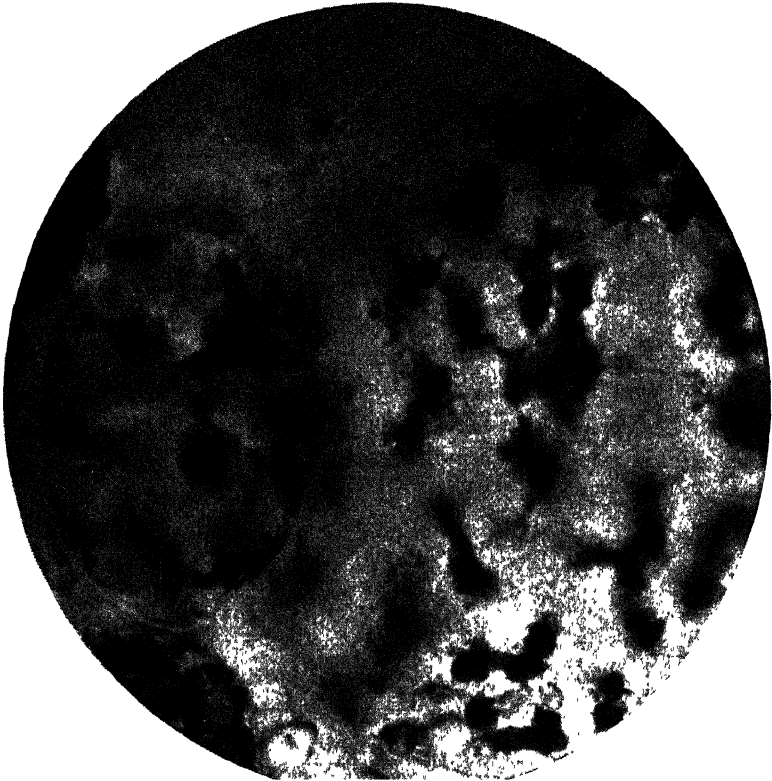


Fig. 2 Diatene stage of *Rhomaleum micropterum*, magnified 3000 diameters. Chromomere vesicles.

The paired threads become looped to form the bouquet stage of the leptotene stage as seen in cell *e* of figure 15. The dense body is the X-chromosome, near which lie close together the distinctly granular-appearing ends of the chromosomes of each pair, while the remaining part of the leptot-

tene chromosomes are bilaterally disposed in symmetrical loops. Another leptotene cell *A* is present in figure 11. That the purpose of the spireme formation concerns an equal division of nuclear entities, supposed to be linearly arranged upon the spireme, was suspected by Balbiani ('81) Pfitzner ('82), Roux ('83), and Van Beneden ('83). Later, De Vries ('03) and Strasburger ('05, '08, '09) maintained that in addition to the alignment of nuclear units upon the spireme thread which would allow its equal division in mitosis, their side-by-side position would allow exchanges of units in the maternal and paternal leptotene threads during the process of maturation.

The observations made by the many careful workers upon fixed and stained material make it certain that the spireme thread is not a continuous thread, but a linear arrangement of smaller elements called chromomeres. Their presence and linear arrangement, evident in the photographs of the living cells in figures 15 and 16 and the diplotene stages on plate 2, fully corroborate the observations made on the organization of chromosomes in the spermatogenesis of certain grasshoppers by Pinney ('08), Carothers ('16), Wenrich ('16), McClung ('99, '00, '27), Bělař ('28, '29), and others.

The vesicles apparent in the cells of figures 32 and 2 are probably chromomere vesicles first described by Carothers ('13) as plasmosome-like bodies related to definite regions of the chromosomes and later ('17) that this relationship pertains to separate chromomeres—hence the name chromomere vesicles. Wenrich ('17) has produced further evidence that the chromomere vesicles are constant both in position and number. The chromomeres are more evident in the stages in which the leptotene threads of each pair are brought together in parasynaptic association for conjugation. As the threads become shorter and thicker the chromomeres remain distinct and lie opposite each other, two by two, as seen in the diplotene stages on plate 2. In the greatly magnified photograph of a diplotene stage, figure 22, one chromosome pair, *a*, shows distinctly the existence of a coiled thread around its periph-

ry. Is this the spireme thread of the earlier stage, figures 5 and 16, becoming wound more closely to form the chromonema of the contracted diplotene chromosomes? It resembles the spiral or zigzag disposition of the chromonema described by Martens ('22) in the seed plant, Paris. According to

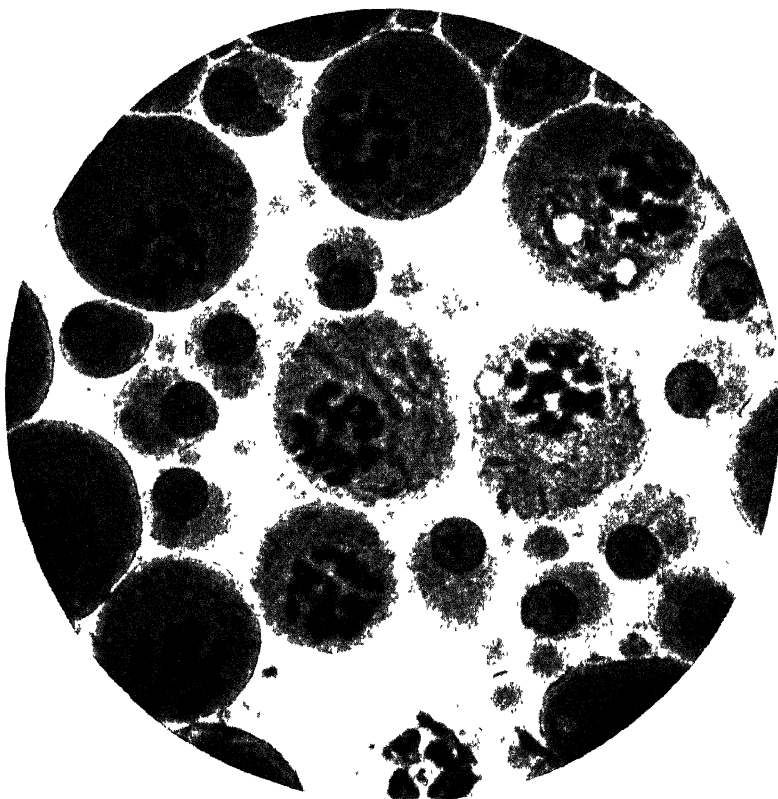


Fig. 3 Tetrad stages of *Melanoplus femur rubrum*, magnified 500 diameters. Mitochondria as filamentous threads in cytoplasm.

Martens, the chromonema concentrates on opposite sides, giving the appearance of longitudinal splitting, the cleft cutting across the turns of the spiral, as is also apparent in chromosome *b* of the same cell (fig. 22). This view is contrary, however, to the observations made by the many careful workers on the spermatogenesis of the grasshopper.

The chromosomes become more and more contracted as the growth period of the primary spermatocytes continues until all are split longitudinally, the maternal and paternal chromosomes of each synaptic pair still remaining together and finally coming out in the form of tetrads as seen in figures 3,

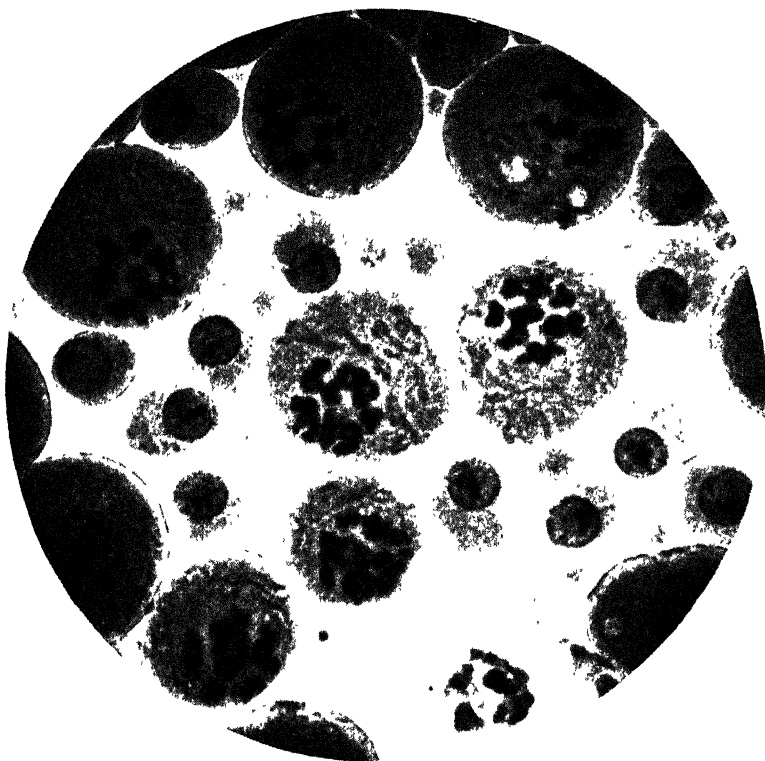


Fig. 4 Same as figure 3,  $2\mu$  lower.

4, and 5. The typical crosses and rings observed by all students of the spermatogenesis of the grasshopper are apparent. In the division that follows, the mates of each pair are distributed to opposite poles, forming the prophase of the secondary spermatocyte in which the chromosomes are already split into longitudinal halves. These separate from one another in the ensuing division, as seen in figures 6 and 7



nd plate 3. The twelve chromosomes at each pole are easily counted in the twelve optical sections through the cell 26 of plate 3, the six sections through the cell 27 of plate 3, and the ten of cell 28, plate 3. The early telophase of cell 29, plate 4, shows the chromosomes in their typical pole-to-pole



Fig. 5 Tetrads of *Melanoplus femur rubrum*, magnified 1800 diameters.

position and still retained in the later stage of cell 30, plate 4, where they are disappearing in their vesicles to form the nucleus of the resulting spermatid.

In the final telophase division, as seen in *M* of figure 8, the chromosomes appear more threadlike. This return of the chromatin to a spireme, one for each chromosome and each in its own vesicle, is distinctly apparent in a still later

stage, a spermatid, beginning to grow a tail as seen in the upper cell (partly off the field) in figure 31.

Equally interesting are the details in the structure of the cytoplasm brought out by this method of photography. In the late telophase stage, as seen in *M* of figure 8, a row of granules between the two daughter cells is apparent, the *Zwischenkörper*. This body is not so well developed in animals as in plants, where it was first observed by Strasburger ('98) to be a product of the spindle fibers and to form the partition wall or cell plate between the two daughter cells. Flemming ('92) was the first to describe it in his study of mitosis in salamander cells. Since then it has been observed by others, but always as a rudimentary structure in animals represented only by a few granules. Since this body is considered a product of the spindle fibers, it is interesting to note that their product is photographed, while the spindle fibers do not register at any stage. They are barely perceptible at both poles of early anaphase stages in the beautiful photomicrographs of living germ cells of *Stenobothrus lineatus* by Bělař ('28, '29).

In the early growth period, as indicated in figures 1 and 8, the mitochondria appear as granules distributed throughout the cell. As growth progresses these are changed to filaments, as is evident in figures 3, 4, and 9. The distribution of the mitochondria in the secondary spermatocyte divisions is not indicated in the photographs. They are noticed, however, as a distinct aggregation in the spermatids of figure 33. In the upper cell of figure 31, many of which were observed 'in vivo,' the mitochondria are passing into the developing tail. Making use of a special technique, Altmann ('70) was first to demonstrate the presence of granules (mitochondria) in cells. He regarded these as the essential units of protoplasm. The fibrillar stage was first observed by Flemming ('82) in living cells. Their existence has been confirmed by many more recent observers. As these photographs indicate, they form the *nebenkern* or the chondriosome body, which later draws out to form the tail envelopes about the tail fila-

ment. This was first noticed by La Valette St. George ('67), although the aggregation of mitochondria going into the tail was not named the nebenkern until 1871 by Bütschli. This behavior of the mitochondria has been studied by many more

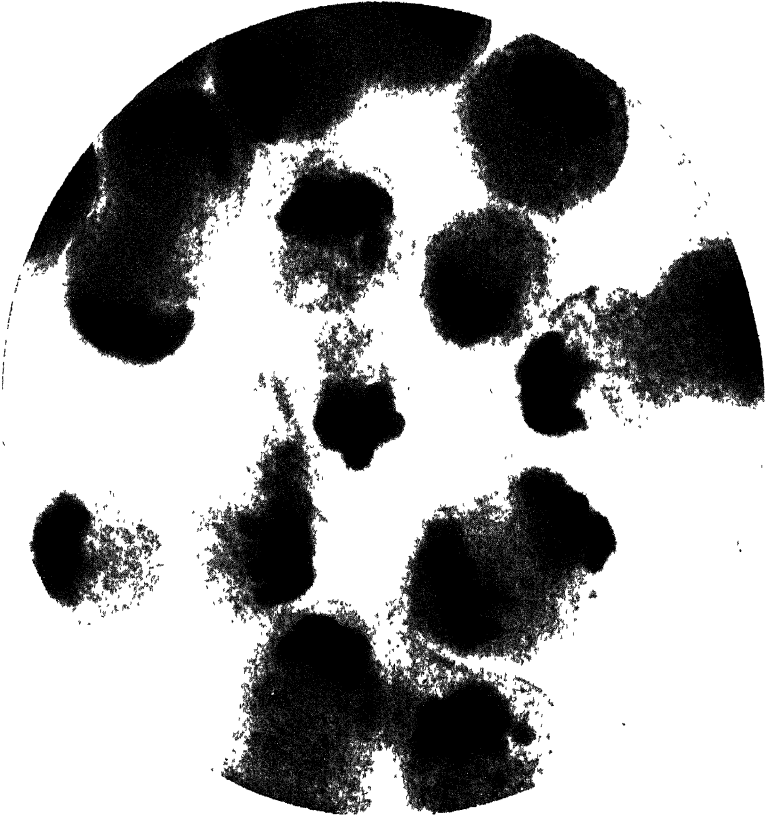


Fig. 6 Secondary spermatocyte divisions of *Melanoplus femur rubrum*, magnified 1800 diameters.

recent investigators in various types of animals. Bowen ('23) has shown that the halves of the nebenkern become drawn out into very thin threads which show at intervals as swellings or 'blebs' that later disappear. These blebs are present in the filamentous forms of the developing spermatozoa in figures 1 and 8.

Bělař ('28) was the first to make photomicrographs of living germ cells of the grasshopper and of the same cells fixed and stained for comparison. Those of the living cells show the chromosomes distinctly—also, the details in splits, chromomeres, and point of spindle attachment, verifying fully the

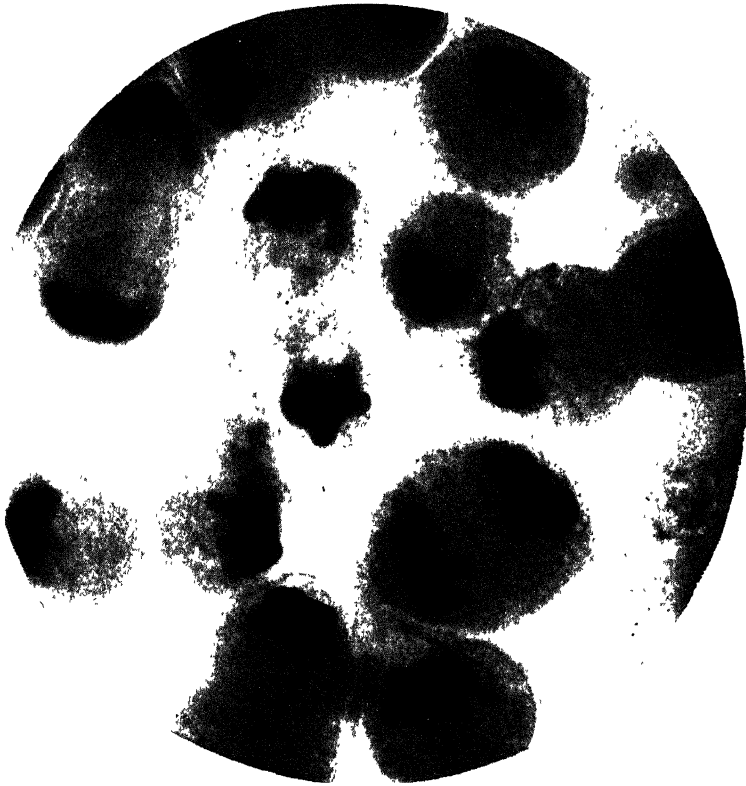


Fig. 7 Same as figure 6,  $2\mu$  lower.

work done by cytologists on fixed and stained material. His photomicrographs of the same cells fixed and stained merely show the same details in much greater contrast. Photomicrographs of living cells with ultraviolet light, as indicated in this paper, bring out the same contrasts as if the material had been fixed and stained. The only difference is the absence of spindle fibers. These, however, show in the ultraviolet-light

hotomicrographs of fixed follicles sectioned but unstained. The desire so often expressed that if one could only observe directly the exact nature of nuclear phenomena in living cells, it would be possible to follow the continuity of chromatin

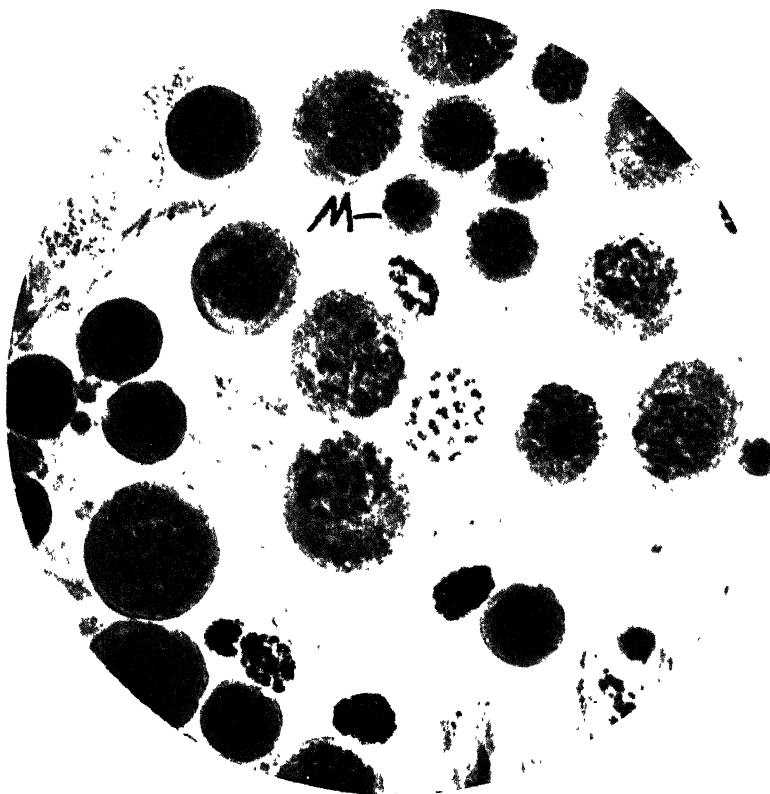


Fig. 8 Sperm cells of *Melanoplus femur rubrum*, magnified 500 diameters. The largest cells are primary spermatocytes with mitochondria in cytoplasm, *M*. One telophase stage of secondary spermatocyte division with Zwischenkörper. Various stages of spermatids, some with blebs.

from one generation of cells to the next is beginning to be realized. The so-called 'optically empty' nuclei of living cells, which have time after time served as evidence that the differentiations brought out by technical methods are artifacts, when photographed with the ultraviolet light are found not to be homogeneous or optically empty, but to contain the

chromatin, even though diffuse, in the form of early diatene chromosomes (pl. 1), thus proving that chromatin, though more liquid or diffuse in some stages, is actually continuous. Photomicrographs confirm the findings of the many observers

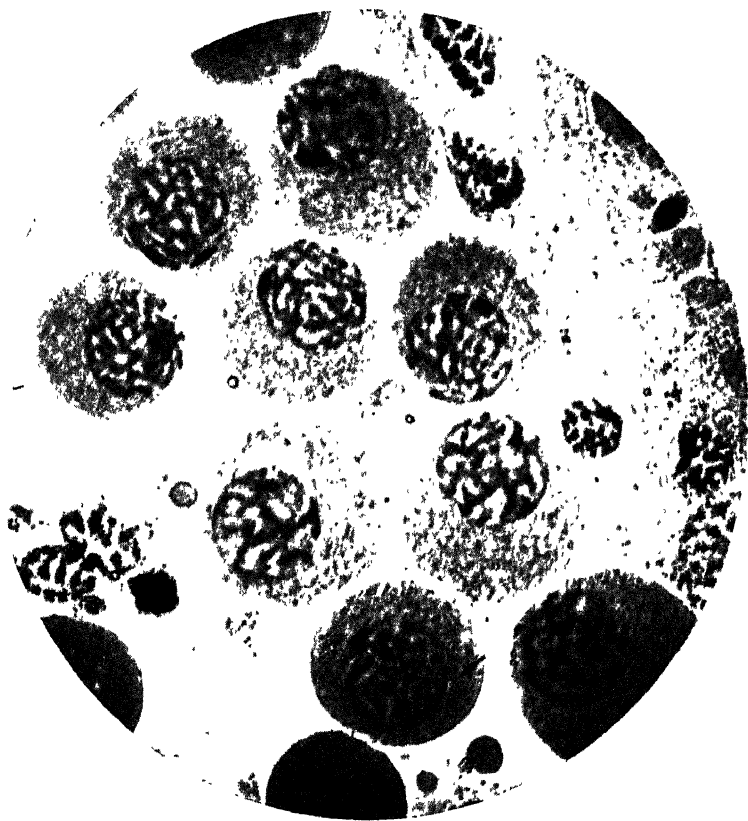


Fig. 9 Primary spermatocytes of *Melanoplus femur rubrum*, magnified 500 diameters. Filamentous mitochondria in cytoplasm.

on the spermatogenesis of the grasshopper—especially, in the spinning out of the chromonema from the solid blocks of the diatene stage, the pairing of same in the leptotene stage, the development of the tetrads, the final distribution of the chromosomes in the resulting spermatids and their return to a spiral chromonema, each enclosed in its own vesicle.

Further development of this method of recording the structures of a living cell in its various phases of activity, it is hoped, will not only enable one to follow more clearly than ever the individuality of a chromosome from one generation of cells to another, but also to pry into the minute structure of the individual chromosome.

Acknowledgments are gratefully made to the Department of Zoölogy, University of Pennsylvania, for specimens of *Melanoplus differentialis*; also, to Miss A. K. Marshall, of the Bell Telephone Laboratories, for much assistance in the preparation of the material.

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## EXPLANATION OF PLATES

All photomicrographs reproduced approximately five-sixths of the original size.

### PLATE 1

#### EXPLANATION OF FIGURES

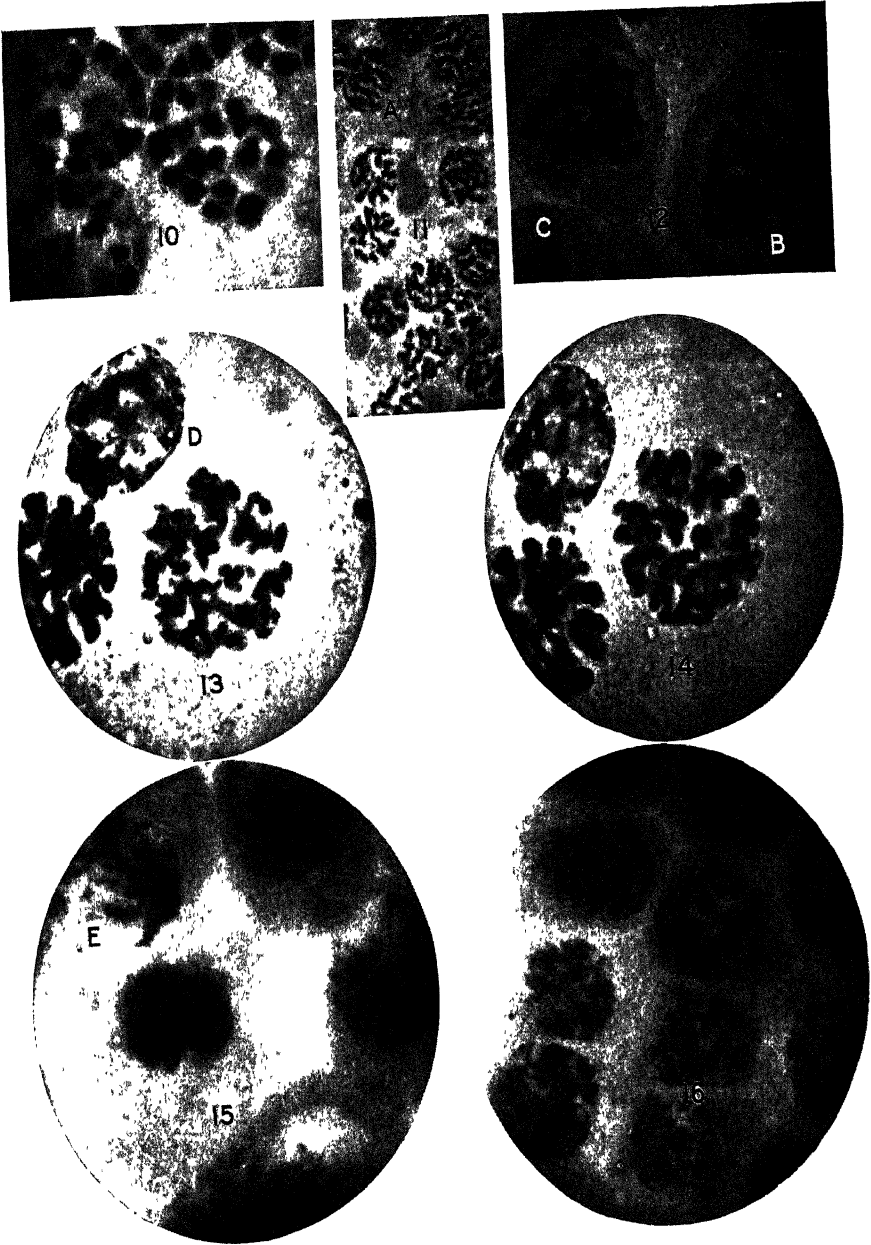
10 Diatene stages of *Melanoplus femur rubrum*, magnified 1800 diameters.

11 Diatene stages of *Melanoplus femur rubrum*, magnified 500 diameters. Cell *A* is a leptotene stage.

12. Diatene stages of *Rhomaleum micropterum*, magnified 1200 diameters. Cell *B*, polar view of X-chromosome; *C*, lateral view showing distinct spiral thread.

13 and 14 Diatene stages of *Melanoplus differentialis*, magnified 1800 diameters. Cell *D* with a vacuolated form of the X-chromosome vesicle.

15 and 16 Prochromosome stages of *Melanoplus femur rubrum*, magnified 1800 diameters. Cell *F*, a leptotene stage showing chromomeres.



## PLATE 2

### EXPLANATION OF FIGURES

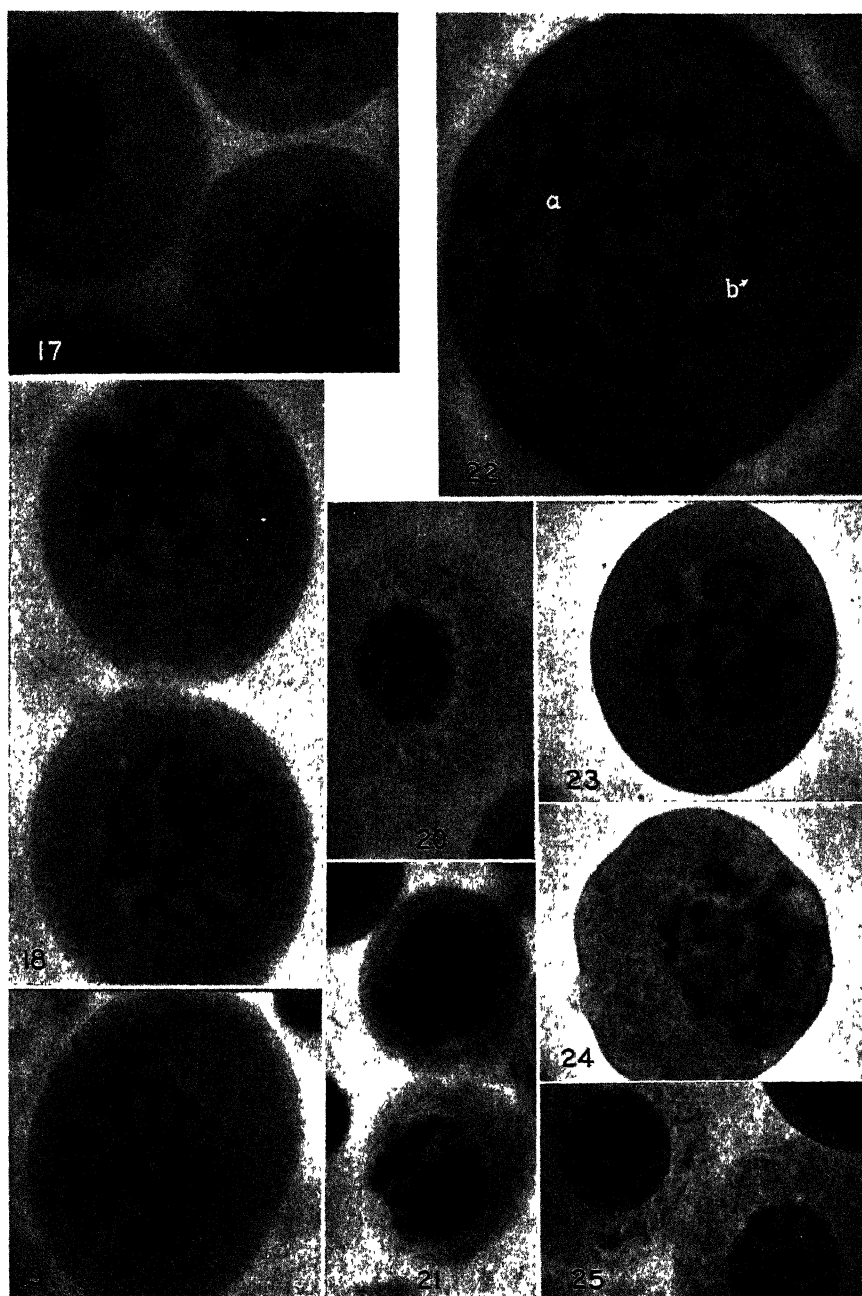
17 to 19 Diplotene stages of *Melanoplus femur rubrum*, magnified 1800 diameters.

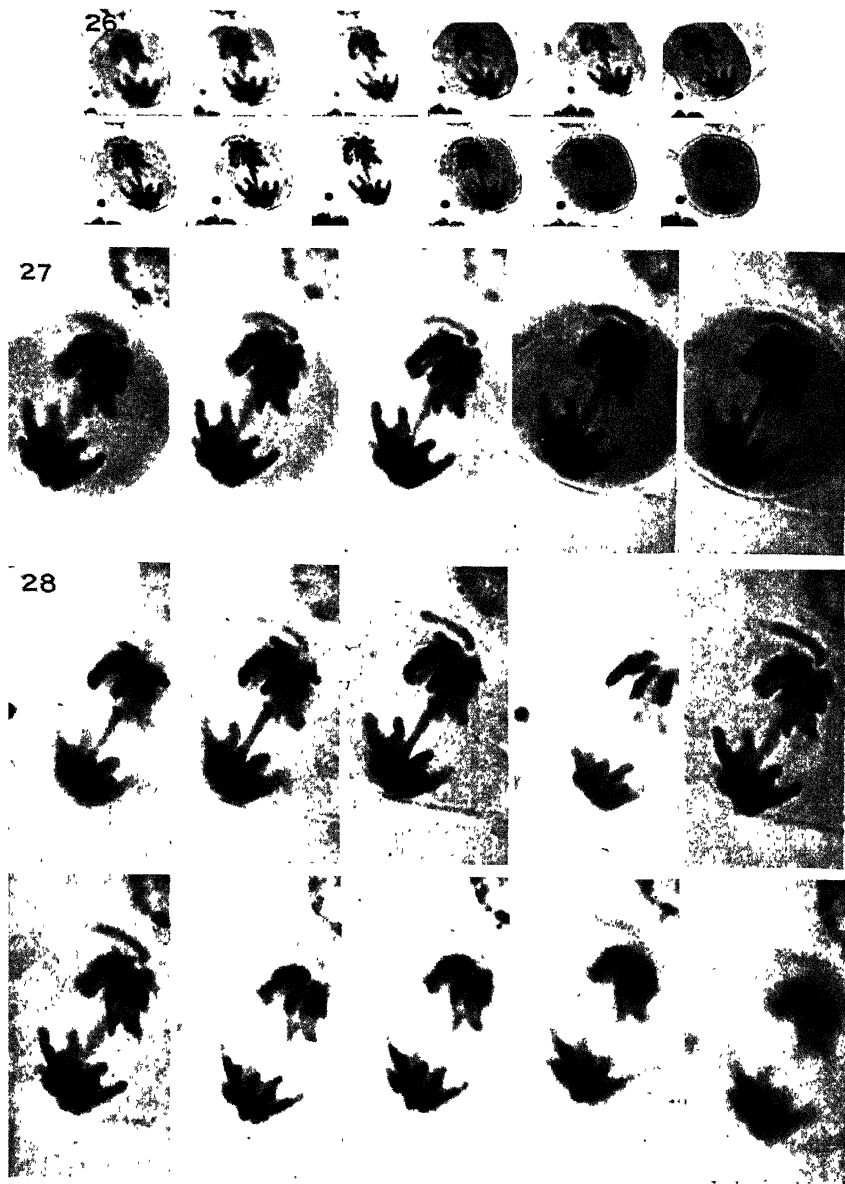
20 and 21 Diplotene stages, magnified 1200 diameters.

22 Diplotene stages of *Melanoplus differentialis*, magnified 3000 diameters.  
Chromosome *a* with zigzag chromonema; *b*, showing longitudinal cleft.

23 and 24 Diplotene stages, magnified 1800 diameters.

25 *Melanoplus femur rubrum*, magnified 1200 diameters.



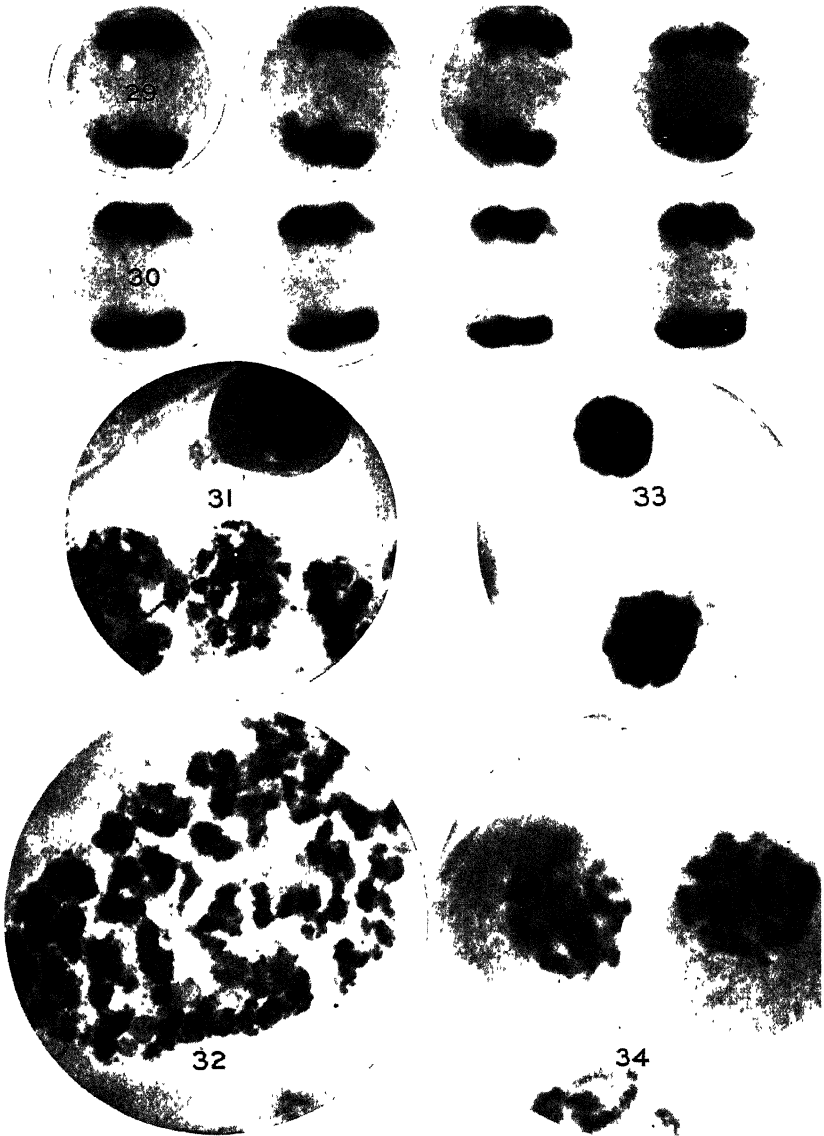


26 Anaphase of secondary spermatocyte division of *Melanoplus femur rubrum*, magnified 500 diameters. Twelve optical sections of one cell.

27 The same, magnified 1200 diameters. Six optical sections.

28 The same. Ten optical sections of one cell.

29 and 30 Telophase of secondary spermatocyte division of *Melanoplus femur rubrum*, magnified 1200 diameters. Figure 30 a little later than 29.



31 Upper cell, spermatid growing tail, *Melanoplus differentialis*, magnified 1800 diameters. Mitochondria passing into tail.

32 Diatene stage of *Rhomaleum micropterum*, magnified 3000 diameters, with chromomere vesicles.

33 Spermatids of *Melanoplus femur rubrum*, magnified 1800 diameters. Mitochondria in cytoplasm.

34 *Melanoplus femur rubrum*, magnified 1800 diameters.



# THE HISTORY OF THE GERM CELLS IN THE VIVIPAROUS TELEOST PLATYPOECILUS MACULATUS

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ONE TEXT FIGURE AND FOUR PLATES (TWENTY-EIGHT FIGURES)

## AUTHOR'S ABSTRACT

This study is based upon a series of ontogenetic stages from just before coelom formation to maturity.

Primordial germ cells are first seen at the outer edge of the lateral mesoderm and are traced from there to a position in the genital anlagen. Counts indicate that most of them succeed in reaching the genital anlagen, where they form definitive reproductive cells in both sexes.

The evidence seems to show that there is some transformation of somatic cells into germ cells in the immature female, but that this transformation is not extensive. In the male the primordial germ cells are the sole source of the definitive elements.

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## INTRODUCTION

The germ-cell cycle in animals has for the past fifty years been a fertile subject for speculation and research. Although some of the theoretical aspects of the problem seem less important to us than they did to the earlier investigators—the question, for instance, of the bearing of a somatic origin of reproductive cells upon the mechanism of heredity—there has nevertheless been a steady increase in the literature of the subject down to the present day. Much more detailed information must be secured before broad generalizations can be made.

Hegner, reviewing the subject in 1914, listed in his bibliography some 450 publications dealing with one aspect or another of the germ-cell cycle. Okkelberg, in 1921, summarized the work on vertebrates and included in his bibliography fifty-six papers. McCosh carried this summary down to 1930 with a review of more than thirty papers published after 1921. Heys again reviewed the whole field in 1931. In view of the thoroughness of these synopses, it seems unnecessary at this time to present any formal review of the literature dealing with reproductive cells. Suffice it to say that the reports of investigators of teleostean germ cells have been as variable and contradictory as those of the workers in any other group of vertebrates.

Of the various theses proposed, however, one deserves special mention, it being one of the more recent attempts to bring order out of the germ-cell chaos. This theory involves a phylogenetic interpretation of the problem of the origin of the definitive reproductive cells in that it accepts primordial

germ cells as being perhaps the only source of the definitive elements in the lower chordates (cyclostomes), whereas the germinal epithelium is the sole source of these elements in the higher forms (mammals). Intermediate groups (teleosts, Amphibia) may have definitive germ cells arising from both sources with the emphasis either way. This theory was formulated as early as 1920 by Firket, and has been propounded recently by several investigators.

Several recent writers have emphasized one obstacle in the way of harmonizing the results of the various workers on vertebrate germ cells, namely, these cells have been studied so frequently only from their first appearance up to the time they reached the germ-gland anlagen. Or, again, the primordial cells have been ignored and only the later stages of germ-cell formation investigated. As late as 1921, Okkelberg could state that there existed no complete account of the germ-cell cycle in any vertebrate. Since Okkelberg's paper on the brook lamprey several other complete accounts have appeared. The writer has been able to find two such accounts bearing upon the teleosts: the work of Essenberg ('23) on *Xiphophorus hellerii* and that of Hann ('27) on *Cottus bairdii*.

The present study was undertaken with the hope of giving a description of the entire germ-cell cycle of another teleost. The form studied, *Platypoecilus maculatus*, belongs to the same family as does *Xiphophorus*, studied by Essenberg. This opens the way for certain significant comparisons.

The writer wishes to express his appreciation to Prof. Hugh D. Reed, at whose suggestion and under whose direction this investigation was made. My thanks are also due to Dr. Myron Gordon for supplying me with most of the embryos used in the study.

#### MATERIALS AND METHODS

*Platypoecilus maculatus* is a cyprinodont of the family Poeciliidae, native to the fresh and brackish waters of southern Mexico. It is a viviparous fish well known to aquarists

under the name of 'platy' and can be obtained in a great variety of color patterns. Under aquarium conditions the females mature in about six or eight months, and thereafter produce broods throughout the year at about thirty-day intervals. The broods in our aquaria have numbered from eight to as many as sixty-four at one bearing.

A series of embryos, young fish, and adults was used in this study, over a hundred animals being sectioned and studied.

A variety of fixers were tried, most of the material finally being fixed in Allen's modification of Bouin's. It was found to be extremely difficult to section the yolk of the young embryos. There was found no better method of obviating this difficulty than cutting away as much of the yolk as possible before embedding. All sections were stained with Heidenhain's iron hematoxylin and counterstained with eosin.

#### THE INDIFFERENT PERIOD

##### *Observations*

a. *1.6-mm. embryo* (fig. 1). Due to the difficulty involved in obtaining a complete series of embryos in a viviparous animal, together with the rather limited source of material available, I was unable to get the cleavage stages, or, indeed, the stages of germ-layer formation. In the embryo here described—the earliest I was able to obtain—the neural tube, notochord, and myotomes have already been formed.

At the outer edge of the lateral mesoderm are a number of cells distinguished from all the other cells of the embryo by their larger size, the indistinctness of their nuclei, and by the presence in some of them of numerous yolk spherules (fig. 1). The more cephalic of these cells lie in the lateral mesoderm, but the more caudal appear to lie out beyond the lateral mesoderm in the extra-embryonic region. Owing to the curvature of the embryo around the egg, the caudal part of the animal—the region of the large cells—was cut about halfway between a trans- and a frontal section. This somewhat obscures the relation of the large cells to the lateral mesoderm in figure 1

and at the same time gives structures such as the neural tube and notochord much greater depth than they really possess. It will be noticed that all the large cells are amoeboid in appearance, one possessing large pseudopodia.

These large amoeboid cells are considered by the writer to be germ cells. Part of the justification for so considering them comes from their later history and so cannot be discussed at this time, but it might be in order to mention that large size and presence of yolk spherules are two of the characteristics of early primordial germ cells mentioned by almost all investigators, and the amoeboid appearance at some stage of their history has been mentioned by many.

*b. 1.2-mm. embryo* (figs. 2 to 4). This embryo, though smaller than the preceding one, is somewhat further advanced in development. The cephalic part of the lateral mesoderm has split to form the coelom, while caudally the lateral mesoderm is still a solid plate, roughly two-layered.

In an embryo of this stage there are found the large cells of the same general appearance and size (table 1) as in the preceding specimen. These large cells have the same cephalo-caudal distribution as in the younger embryo, but occupy a more median position. They are arranged in two bands which converge anteriorly so that the more cephalic of the germ cells lie chiefly within the undivided lateral mesoderm, whereas the most caudal cells lie out beyond the lateral mesoderm in the extra-embryonic region, between ectoderm and periblast (figs. 2 to 4).

Large primordial germ cells in the solid plate of lateral mesoderm similar to that shown in figure 3 have been reported by Eigenmann ('92) in *Micrometrus*, Woods ('02) in *Squalus*, Allen ('11) in *Amia*, Richards and Thompson ('21) in *Fundulus*, Okkelberg ('21) in *Entosphenus*, and Butcher ('29) in *Petromyzon*. Hann ('27) reports in *Cottus* a condition differing in detail, but essentially similar to that described here. So it would seem that an early appearance of primordial germ cells in the lateral plate is quite a usual condition in fishes.

*c. 1.8-mm. embryo* (figs. 5 to 8). When the embryo has reached this size, the wolffian ducts have formed, as well as the intestine.

Figure 6 shows a cross-section through the middle of the sex-cell region. It will be seen that the germ cells are closely grouped immediately ventrad of the wolffian ducts. They lie thus in two bands beneath the wolffian ducts for some distance cephalad and caudad of the section here shown.

Figure 7 shows a section toward the caudal end of the germ-cell bands. Two germ cells are seen here occupying a position laterad of the wolffian duct. In addition, there is another germ cell lying much farther out to the right in the lateral mesoderm (torn in this section). This cell does not have so smooth an outline as the two more median cells. All three possess coarse nuclear granules, and the nuclear membrane is much less distinct than in the more cephalic cells of the germ bands.

The next section caudad of the one described above contains two similar cells on the other (left) side of the embryo (fig. 8). On this side there are no germ cells ventrad of the wolffian duct. One of the two cells out in the lateral mesoderm is barely included in the section, which accounts for its smaller size and rather vague outline. The other shows the same large granules as did the cells of the preceding section. None of the sections caudad of this have any sex cells under the wolffian ducts, but several have large cells out in the lateral mesoderm.

It might be well to present the evidence for the identification of these cells. That these large laterally lying cells are identical in type with the large 'germ cells' of the two younger embryos already discussed is something more than suggested by comparing figures 2 and 4 with figures 7 and 8. It is readily recognized that the large subwolffian-duct cells shown in figure 6 are true primordial germ cells. Their size, appearance, and position agree so well with the primordial germ cells of so many other vertebrates at a similar stage of development that it seems unnecessary to cite any particular

investigation for corroboration. It has already been pointed out that as these two bands of germ cells extend caudad they swing out to the sides, so that the most caudal of the unmistakable germ cells (fig. 7) lie laterad of the wolffian ducts. Still farther caudad lie a few scattered cells very much like the sex cells in appearance, but decidedly more lateral in position (figs. 7 and 8). Figure 5 is a drawing of a dorsal view of this embryo showing the approximate location of the reproductive cells. In this figure the cells have not been placed as a result of any accurate measurements; both their cephalocaudal and mediolateral distribution is only roughly shown.

As stated before, the writer takes all the large cells here described, including the most caudal and lateral, to be germ cells. The embryo differentiates in a cephalocaudal direction, so that the anterior region shows a more advanced state of development than does the posterior. If all the sex cells originated at the lateral edge of the lateral mesoderm, they would wander, or become shifted mesiad into the embryo, the more cephalic cells first. Thus at all stages up to the time this migration had been completed the germ cells would lie in two lateral bands which approximate one another more cephalically than caudally. This has been the case in all the embryos so far discussed.

It might be objected that the most caudal of the large cells in the 1.8-mm. embryo (fig. 8) are not germ cells, for they differ from those under the wolffian ducts (fig. 6) in appearance. But this difference is due to an earlier embryonic state of the caudal cells. The caudal cells contain yolk platelets and an obscured nucleus—two characteristics very generally attributed to early primordial germ cells. Furthermore, the large caudal cells correspond in size with the germ cells (fig. 6), no other cells in the entire embryo approaching them in this respect.

The V-shaped position of the germ bands has been noticed in the embryos of other species. Richards and Thompson ('21) state concerning a *Fundulus* embryo in a corresponding period of development:

The position of the sex-cells at this period is most striking. There is a wide range of distribution in each embryo. The most anterior of the sex-cells were invariably farther along the germinal path than were the most posterior ones of the same embryo.

Their work contains two figures exhibiting the condition shown in figure 5. Both Okkelberg ('21) and Butcher ('29) report the same condition in lampreys.

Dodds ('10) gives outline drawings of embryos of *Lophius* at four different stages, showing the number and position of the reproductive cells. These figures do not show any such distribution of cells as do those of Richards and Thompson or my figure 5. It is possible that he missed the critical stages or that he overlooked the lagging of the caudal sex cells.

Thus, in view of their position, as well as size and appearance, it seems correct to identify the large cells shown in figure 8 as 'germ cells.' For the same reason the large cells of the two earlier embryos are considered as belonging to the same category.

*d. 2-mm. embryo* (fig. 9). In this embryo the two bands of germ cells have shifted medially until they form a single median ribbon below and between the wolffian ducts at the base of the rudiment of the dorsal mesentery (fig. 9). The same force which causes the pulling down of the intestine and consequent elongation of the dorsal mesentery is probably responsible for the shifting inward of the germ cells from their position beneath the wolffian ducts to their new median position between these ducts. Some of the cells bulge out into the coelom on each side of the mesentery, this bulge being the beginning of the germ-gland anlagen.

Some of the germ-cell nuclei are rather irregularly lobed, though this does not show in the particular section here figured. The cell outline is in all cases quite smooth and regular. It will be recalled that in earlier stages some of the sex cells were quite amoeboid in appearance.

So far no mention has been made of the number of germ cells present in the embryos. This particular embryo possessed ninety-nine cells. It will be seen from table 1 that

the earliest stages contained about fifty such cells. About the time the cells reach a position near the germ-gland anlagen the number of cells is roughly doubled. The whole question of multiplication of the germ cells will be discussed at length further on.

*e. 3.5-mm. embryo* (fig. 10). This may be described as the genital-ridge stage. The yolk sac has almost absorbed. The germ cells are once more confined to two longitudinal ridges—the germ-gland anlagen—lying almost exactly below the wolffian ducts and projecting downward into the body cavity (fig. 10).

The germ-gland anlagen now contain three kinds of cells: first, the large distinctive germ cells; secondly, the peritoneal cells bounding the anlagen; thirdly, cells scattered in among the germ cells in the body of the gland—the loose cells which will be referred to as stroma cells. The nuclei alone of these two somatic constituents of the germ-gland anlagen are visible.

The stroma cells have been claimed by Eigenmann, Essenberg, and Hann—all working on teleosts—to originate from the peritoneum. Both Okkelberg and Butcher in lampreys derive at least a part of the stroma from the mesenchyme lying dorsal to the germ glands. McCosh, in the amphibian *Amblystoma maculatum*, interprets the stroma cells as coming from the “mesenchymal tissue of the mesonephric region.” Though this is perhaps not an important point, it certainly seems as if the condition in *Platyopocilus* agrees better with that found in lampreys and *Amblystoma* than with the condition reported for other teleosts. A glance at figure 10 shows that the somatic stroma cells of the germ-gland anlagen differ in no way from the mesenchymal cells dorsal to the glands. There is no reason for assuming that they have a purely peritoneal origin.

*f. 5.6-mm. embryo* (fig. 11). The embryos here under consideration are in the last stages of prenatal development. They are still curled up in the follicular membrane. Upon removal from the body of the parent and the rupturing of



this membrane, they are capable of swimming. The yolk sac is almost completely absorbed. A cross-section through the gonad region shows large quantities of undigested yolk within the body cavity. The germ-gland anlagen are similar in position to those in the previous embryo, but protrude much farther into the coelom (fig. 11). In consequence of the development of the air bladder—not yet formed in the 3.5-mm. embryo—the anlagen have been pushed downward away from the notochord and wolffian ducts. The peritoneum from which the gonads are suspended now forms part of the ventral wall of the air bladder.

The condition of the gonads at this time resembles closely the corresponding stages described by Eigenmann ('96) for *Cymatogaster*, Essenberg ('23) for *Xiphophorus*, and Geiser ('24) for *Gambusia*. The same elements are present as in the germ-gland anlagen of the previous stage, i.e., large, unmistakable germ cells and much smaller stroma and peritoneal cells. As in the previous stage, these two latter types are indistinguishable. Stroma and germ cells are evenly distributed through the gonad. Blood vessels are beginning to make their appearance.

The embryo figured contained 223 germ cells. The increase in the number of germ cells at about this time is shown in table 1. Mitoses are common in some gonads, affording direct proof that the germ cells are dividing.

Since many embryos in the neighborhood of 5 mm. in length show such a decided increase in the number of sex cells (tables 1 and 2), the question arises whether there is not some transformation of stroma cells into germ cells at this stage. All the embryos were examined critically for evidence of such transformation, but the findings appear to be negative. This transformation is a difficult point to settle. Germ cells differ from stroma cells in being of larger size, possessing visible cell membranes, and containing much more chromatin material in the nuclei. In general, the stroma nuclei are more spindle-shaped than are those of the germ cell. If any one of these factors is taken by itself, it is possible in many

instances to find intermediate cell types. But, considering all the characters together, these somewhat intermediate types are hardly convincing evidence of transformation. Another point to consider is that invariably the gonads containing the most of these so-called intermediate cells are gonads in which the germ cells are dividing actively. Thus their nuclei present a great variety of appearances and even of sizes, for the recently divided cells are always somewhat smaller than the rest. The presence of these smaller germ cells increases the chance of finding intermediate cells.

Since the transformation of somatic cells into germ cells is one of the moot questions in the whole germ-cell problem, the material was examined again and again in order to determine this point. At times the writer was almost convinced that such transformation does occur, but reexamination of the material after an interval of several days always raised doubts. The most emphatic statement on the subject that the writer feels able to make is that he is strongly of the opinion that up to the end of the indifferent period in the development of the gonads no transformation of somatic cells into germ cells takes place.

### *Discussion*

*a. Place of origin of primordial germ cells.* In the earliest embryo obtained the sex cells were located along the outer edge of the lateral mesoderm and even apparently out beyond it, so that isolated cells appear to be lying between ectoderm and periblast. These cells might have been part of the mesoderm, or they might have originated from the ectoderm, or they might even have come from the periblast, although the first alternative seems most likely. Each of these regions and, in addition, the ectoderm have been claimed the place of their origin by various workers upon fishes.

A majority of the recent workers on teleosts have distinguished the first germ cells among the cells of the entoderm (Allen, '11; Richards and Thompson, '21; Hann, '27). Some have found them in the mesoderm (Bachmann, '14).

Dodds ('10) discovered the earliest sex cells in the combined ecto-endoderm before these two layers had separated. One worker (Reinhard, '24) first saw them in the form of 'giant cells' in the periblast.<sup>1</sup> One is unable to see how any of these results, with the possible exception of Reinhard's, conflict fundamentally. The important fact brought out by all these recent investigations is that the germ cells of teleosts are segregated early, though not in the cleavage stages. In which germ layer and at just what stage in the ontogeny of the embryo the sex cells first become distinguishable from somatic cells is hardly of secondary importance.

*b. Migration of germ cells.* The amoeboid appearance of the early primordial germ cells of *Platyopocilus* seems to justify the conclusion that their first change in position, that which carries them into the lateral mesoderm, is an active amoeboid migration. All subsequent movement could be accounted for by the growth and shifting of tissues within which they lie.

In figure A is shown a diagram of the entire migration route of the sex cells of *Platyopocilus*. That part of the movement which is active is shown in dotted lines; the passive movement, in solid.

The idea of an amoeboid movement of the early germ cells of fish has been held by a number of workers. Balfour ('78) suggests the possibility of such a migration. Eigenmann ('96) states positively that the reproductive cells of *Cymatogaster* migrate backward in early stages. Woods ('02) maintains that in *Squalus* the germ cells migrate through the tissues. Beard ('04) is convinced of amoeboid movement of the germ cells of *Raja batis*. Dodds ('10) declares that in *Lophius* both active and passive change of position seems to be at work. He also states that during most of the period of migration the germ cells are decidedly round in outline.

<sup>1</sup> Hann ('27) speaks of the germ cells of *Cottus* being derived from 'giant cells,' and although he cites the earlier work of Reinhard ('24), he apparently does not apply the term 'giant cell' to the same structure as did Reinhard. Hann's 'giant cells' have a totally different origin from those of Reinhard.

Contrariwise, Richards and Thompson ('21) assert that in *Fundulus* "Migration is passive, being due to forces of growth which are altogether external to the cells themselves."

Regardless of the method of migration, the majority of investigators report an arrival of the sex cells in the lateral mesoderm before this plate splits into somatic and splanchnic layers. When the splitting of the mesoderm takes place, the germ cells can follow three possible courses: 1) adhere to the somatic layer, 2) adhere to the splanchnic layer, 3) maintain a medial movement which results in their becoming incorporated in the median group of mesodermal cells where the somatic and splanchnic layers meet.

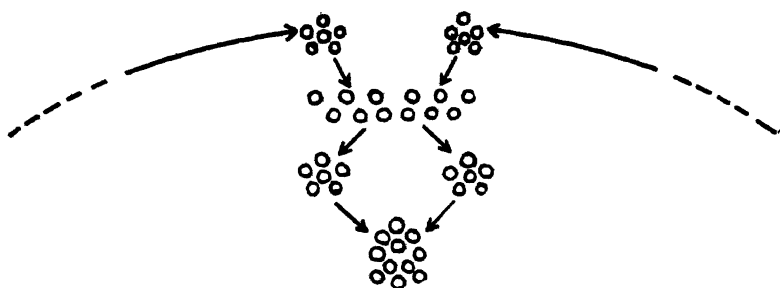


Fig. A Diagram of path of migration of germ cells of *Platyopocilus*. Dotted lines represent amoeboid movement; solid lines, movement due to shifting of tissue within which the germ cells lie.

These three possible courses of the germ cells will result in at least two different methods of approach to the germ-gland anlagen. If they adhere to the somatic layer, they will approach the wolffian ducts from a lateral direction. If they adhere to the splanchnic layer, they may be carried into the peritoneal covering of the gut and so must come up the dorsal mesentery to reach the gonad anlagen. If they adhere to the median portion of the mesoderm, they will probably be incorporated into the dorsal mesentery at the time of its formation and so again reach the gonad anlagen from below.

It is also possible that some cells might adhere to the somatic and some to the splanchnic layers, thus eventually

approaching the wolffian ducts both from the sides and from the mesentery.

In *Platypocilus* the sex cells adhere to the somatopleure and so approach the germ-gland anlagen from the sides. No sex cells have been found in the splanchnopleure. All of the routes indicated above have, however, been reported in various fish.

*c. Multiplication and degeneration of germ cells.* Table 1 presents a numerical summary of the entire indifferent period of gonad formation in *Platypocilus*. The earliest stages in this table contain from forty to fifty sex cells. These undergo no division until they reach the neighborhood of the wolffian ducts. Thereafter they apparently undergo as many as three or four divisions, so that an embryo about to differentiate sexually may contain over 500 germ cells. In some cases, however, almost no division of the sex cells occurs, and so embryos may be found in any stage of development containing but few more cells than do the youngest.

Some of the older embryos show a certain amount of degeneration among the germ cells. In no case, however, did this degeneration appear extensive enough to support any theory of a general degeneration of the primordial germ cells prior to sex differentiation.

It will be noticed that the older embryos (6 to 6.5 mm.) can be placed in two classes as regards number of germ cells: those having 200 or more cells and those having 100 or less. This difference in the number of germ cells, as will appear later, is the first sign of sex differentiation. Those having the greater number of cells are females, and those having fewer cells are males. A difference in cell numbers in the two sexes at this stage was noticed by Essenberg ('23) in *Xiphophorus*, although he did not make actual counts of the cells. This criterion for separating the sexes was not used, although there seems to be no doubt of its validity, because it was felt that the observations made did not include a large enough number of individuals to render it conclusive in every case. Infallible criteria for distinguishing the two sexes appear in slightly older animals.

TABLE 1

*Number and size of the germ cells of Platypoeilus at stages preceding sexual differentiation. The embryos are arranged according to degree of development, regardless of size*

SERIES	LENGTH, MM.	NUMBER OF GERM CELLS	SIZE OF GERM CELLS, MICRONS	SIZE OF GERM-CELL NUCLEI, MICRONS
910	1.6	52	12 × 9.5	6.5 × 4.5
906	1.2	50	11.5 × 8	Indistinct
909	1.7	40	11 × 8.6	6 × 4.3
908	1.8	43	13.4 × 9.9	6.3 × 5
915	1.9	96	12.2 × 8.5	6.1 × 4.2
938	1.5	94	11 × 7.8	6 × 4
911	2	126	11.6 × 8.2	6.5 × 4.4
912	2	99	11.8 × 8.5	6.2 × 4.4
914	2.3	144	11.6 × 8.5	6.1 × 4.1
933	2.5	92	12.6 × 8.4	6.3 × 4.4
923	2.5	89	9.5 × 7.7	5.8 × 4.3
931	2.7	84	9.7 × 7.8	5.6 × 3.9
892	3.5	56	12.7 × 10	6.6 × 5.2
897	3.5	66	11.4 × 9.7	6.3 × 5
895	3.5	77	12.2 × 9.3	6.5 × 5
921	5	151	Not measured	Not measured
871	5	223	Not measured	Not measured
872	5	209	Not measured	Not measured
877	5	130	Not measured	Not measured
918	4.5	463	Not measured	Not measured
898	6	206	Not measured	Not measured
916	4.5	110	11.3 × 9	7.1 × 5.9
917	4.2	83	9.9 × 7.7	6.4 × 5
900	6	65	Not measured	Not measured
899	6	88	Not measured	Not measured
N1	6.5	97	Not measured	Not measured
N2	6.5	69	Not measured	Not measured
N3	6.5	236	Not measured	Not measured
N4	6.5	543	Not measured	Not measured
N6	6.5	183	Not measured	Not measured
N7	6.5	91	Not measured	Not measured

TABLE 2

*Number of germ cells in female Platypoecilus gonad at stages following sexual differentiation. The animals are arranged according to degree of development, regardless of size*

<i>Series</i>	<i>Length, mm.</i>	<i>Number of germ cells</i>
919	4.5	500
920	4.5	500 <sup>1</sup>
927	5	453
902	6.5	300
890	6	349
649	5	270
887	7	205
884	12	558
876	16	476
882	17	670
885	24	4225

<sup>1</sup> Estimated.

TABLE 3

*Number of germ cells in male Platypoecilus gonad at stages following sexual differentiation. The animals are arranged according to degree of development, regardless of size*

<i>Series</i>	<i>Length, mm.</i>	<i>Number of germ cells</i>
903	6.5	96
891	6	48
889	6.5	26
2677	7	Hundreds
2678X	7	Hundreds
2678Y	7	Hundreds
922	9.5	1187
874	16	Thousands
881	19.5	600

## FEMALE GONAD

*Observations*

*a. 6 to 7 mm. Early postnatal stage (fig. 12).* This stage represents the condition found in young fish a few days after birth. The originally paired gonads are fusing medially, the process beginning at the cephalic end.

The germ cells have multiplied and are not confined to the periphery of the ovary, but are spread throughout the body of the gland. While both these are female characters for this stage of development, the one indisputable character which fixes the sex of the animal—barring the possibility of sex reversion—is the fact that some of the germ cells have enlarged to two or three times their original size. These are oocytes. Some of the stroma cells have pressed around the oocytes—the first indication of follicle formation (fig. 12).

At about this time the ovarian cavity begins to form. It appears first as two separate cavities, one in each of the gonads. As the process of fusion of the gonads progresses these cavities also fuse. The wall between them gradually breaks down, resulting in a single cavity running the length of the ovary and continuous caudally with the oviduct.

A detailed account of the formation of the ovarian cavity will not be given at this time. Suffice it to say that, as a result of an invaginating process, this cavity becomes at least partly lined by the peritoneal covering of the gonads.

This again introduces the difficult question of transforming stroma cells. Do the stroma cells transform into germ cells at this stage? *Platyplecillus* preparations were examined again and again to determine this point, but, though intermediate cell types were found, the evidence did not appear convincing either way. Finally, preparations were made of two other species of fish belonging to the same family, *Lebistes reticulatus* and *Xiphophorus hellerii*, and these were studied as a check for the observations on *Platyplecillus*. The author's conclusions are that at this stage there appears to be some evidence of transformation of stroma cells into sex



cells, but that this transformation, if such it be, is not very extensive. A great many cases which might at first glance be taken for transforming stroma cells are undoubtedly merely recently divided germ cells.

*b. 7-to-12-mm. fish. Formation of germinal epithelium (fig. 13).* Figure 13 shows a section through an older ovary in which almost no trace of the bilateral origin of the gland can be seen. Some of the oocytes have grown considerably, though the majority have retained their original size and through active division have formed many separate clusters toward the center of the ovary (not shown in this figure). Owing to the great growth of the oocytes, the whole ovary has increased markedly in size, with the result that the stroma between the oocytes is slight. Many of the stroma cells enter into the formation of follicles around the oocytes and many are incorporated within the epithelial wall of the ovarian cavity, so that there remains very little loose stroma among the oocytes. This scarcity of stroma is characteristic of all later stages in the ontogeny of the organ.

The evidence for transformation of somatic into germ cells is a little better at this stage than at any preceding period. The intermediate cells are no longer found in the stroma, as in younger specimens, but in the wall of the ovarian cavity, that is, the 'germinal epithelium.' This epithelium, according to my observations, consists of peritoneal cells to which have been added stroma cells from the body of the ovary. If in the younger stages stroma cells were really transforming into germ cells, it would not be strange if they continued this process after being incorporated within the germinal epithelium. On the other hand, it is possible that only the peritoneal constituents of the germinal epithelium become germ cells and that the stroma cells at no time undergo such transformation. This second alternative seems less probable to the author than does the first.

*c. 24-mm. female, almost mature (figs. 14 to 20).* The ovary at this stage is an unpaired organ lying in the dorsal part of the body cavity just beneath the air bladder, from which it is suspended by a thin fold of mesentery (fig. 14).

In the center of the ovary, and traversing its entire length, is the ovarian cavity. This cavity is continued beyond the ovary, caudally, as the oviduct. It is surrounded by a thick germinal epithelium. Outside of the epithelium, though intimately associated with it, are the oocytes in various stages of growth. These fill out the bulk of the ovary. Between the oocytes is a thin scattering of stroma cells, nowhere numerous or conspicuous. The entire gonad is surrounded by a thin layer of flattened epithelium (fig. 14).

The epithelium of the ovarian cavity, in its definitive state, is an irregular layer of varying thickness, consisting, usually, of a single layer of columnar cells. The cell membranes do not show in my preparations, but the nuclei stand out clearly. These nuclei are mostly spindle-shaped, and contain one, or sometimes two, excentrically placed nucleoli (figs. 15, 20).

Among the spindle-shaped nuclei, however, are found some oval, or even circular, in outline. Some of these oval and circular nuclei have denser protoplasm and a larger nucleolus than the typical epithelial nuclei. These are interpreted as epithelial cells in an early stage of transformation into germ cells. Others, in addition to the above characteristics, have scattered bits of chromatin material in the nucleus. In some of these the cell membranes stand out clearly. Such cells have progressed a little further in the direction of sex cells.

The next step consists in an extrusion of some of the chromatin particles from the nucleus into the cytoplasm. Cells in this stage show a distinct cell membrane and a cluster of chromatin particles just within or just without the nuclear membrane.

In the final stage the nucleus presents a slightly reticulated appearance. It stains less heavily than the cytoplasm. Nucleus and cell membrane have enlarged slightly. The cells now have quite a different appearance from the neighboring cells of the germinal epithelium and are unmistakably oogonia (fig. 20).

All the stages above described are found embedded in the germinal epithelium. As the oogonia are formed they are

crowded into the body of the ovary, though occasionally oogonia are found so deeply embedded in the epithelium as to be in contact with the ovarian cavity (figs. 16, 18).

Cells intermediate between epithelial and germ cells are still difficult to find in *Platypoecilus* at the stage of development here described. In the earlier stages they are even more scarce and in fully mature individuals they seem to be entirely absent. In this connection, it is not out of place to quote the statement of Philippi ('08):

Sehr richtig bemerkt Felix, (in O. Hertwig's Handbuch) dass die Tatsache des zeitlich eng begrenzten Auftretens dieser Neubildung von Genital- aus Cölozmzellen, ohne weiteres die Zahlreichen negativen Befunde erklärt.

The ova which are crowded into the body of the ovary remain in contact with the germinal epithelium. Here they undergo repeated mitoses, forming nests of oocytes identical with those descended from primordial germ cells (figs. 14, 15). All the cells in a nest are in about the same stage of development at first. As the oogonia are pushed out of the epithelium they carry along with them some of the epithelial cells, which can be seen later pressed around the outside of the nest of oocytes and even scattered among them (fig. 15). These epithelial cells finally become the follicle cells of ova.

One, or perhaps several, of the oocytes from a nest now enter upon a period of growth, so that one finds nests in which one or two cells are much larger than the rest. As individual oocytes begin to enlarge the nests as a whole often split until it is difficult to determine from which group of oocytes any particular ovum is derived.

The enlarging ova always remain in contact with the epithelium of the ovarian cavity at some point. Some have a large area in contact, but in most cases contact is maintained at this stage by a bulging of the epithelium or even by a marked pedicle of epithelial cells. The connection is, of course, not visible in every section, but some examples can be seen in figure 14. A neck of epithelium is shown in figure 20. This neck also contains a newly transformed oogonium.

Philippi ('08) reports that in *Glaridichthys* the ova also invariably touch the germinal epithelium. He noticed that at the point of contact there was always present in the epithelium a notch through which the sperm enter from the ovarian cavity to fertilize the egg. The writer could find no such notch in *Platyopocilus*. This may have been due, however, to a lack of the right stage. The problem of the method of fertilization of the egg was not investigated further.

*d. 34-mm. mature female* (fig. 21). The description of the 24-mm. specimen requires but few changes to fit that of a mature animal.

There are no nests of oocytes such as were present in the younger ovary. In the absence of any marked evidence of degeneration, one might assume that all the oocytes have changed to ova. This is hardly possible, however, for it will be noted that the 24-mm. specimen contained over 4000 germ cells (table 2), and while the germ cells were not counted in any older specimen, it is certain that the older fish do not contain such an enormous number of ova. Furthermore, in our aquaria at least, no fish produces anywhere near 4000 offspring in the course of a lifetime. Some of the disparity between the number of sex cells in an immature animal and the number of offspring produced is doubtless due to degeneration of oocytes or ova in the mature fish.

The epithelium of the ovarian cavity no longer functions as a germinal epithelium, for it contains no more transforming germ cells. Thus, such transformation of somatic cells into sex cells as occurs must be limited to immature fish.

Follicle cells have multiplied around the largest of the yolk-free ova and have given rise to a loose ring of large irregular spherical nuclei which have pushed down into the peripheral region of the cytoplasm.

The cytoplasm of the largest of the yolk-free ova has a much more coarsely reticulated appearance than in the 24-mm. specimen.

The most striking difference between the immature ovary and that of the mature fish is due to the fact that some of the

ova in the latter have become filled with yolk (fig. 21). The tremendous expansion of the ova accompanying the deposition of yolk material has nearly occluded the ovarian cavity. In some parts of the ovary it is difficult to locate.

### *Discussion*

In the foregoing pages an attempt has been made to trace the germ cells from their earliest appearance as primordial sex cells to the formation of definitive ova. In every specimen examined germ cells have been present. Some specimens exhibited a certain amount of degeneration among these cells, but in no case did this degeneration seem extensive enough to have any great significance. There is, therefore, an uninterrupted progression from primordial sex cells to definitive ova. Not all the definitive ova can be traced back to primordial germ cells, however. At about the time when sex may be determined some of the stroma cells appear to be transforming into germ cells. Many of the stroma cells later become incorporated into the germinal epithelium, and from that region the transformation into sex cells continues until the animal is almost mature. No transformation was observed in mature animals, and at no time was the transformation very extensive. The definitive ova, therefore, have a double origin: the greater part descending from primordial germ cells, the rest from the somatic stroma cells of the gonad and perhaps from epithelial cells of peritoneal origin.

It seems strange that in as closely related a genus as *Xiphophorus* all the primordial sex cells should disintegrate and that definitive ova should come entirely from peritoneal cells (Essenberg, '23). Essenberg studied cytologically some 300 fish, of which, from the nature of his investigation, probably the majority were females. While the author sectioned and studied over a hundred animals, only about six of these were females falling within the developmental limits set by Essenberg for the period of degeneration of the primordial germ cells. In none of these, however, was there discovered anything resembling an extensive degeneration of these cells.

If they all degenerate in *Platyopocilus*, as in *Xiphophorus*, the examination of a single specimen of the proper age ought to have given some indication of the fact.

It seems, therefore, that *Platyopocilus* and *Xiphophorus*, though closely related, differ greatly in the history of the germ cells in the females. In *Platyopocilus*, possibly, the more primitive condition persists in which primordial germ cells give rise, along with germinal epithelial cells, to definitive ova. In *Xiphophorus* the less primitive condition of definitive ova derived only from germinal-epithelium cells obtains.

#### MALE GONAD

##### *Observations*

*a. 6 to 7 mm. Early postnatal stage.* At about the time that an increase in the number of germ cells in the gonad gives the first clue to the development of femaleness, the gonads destined to become testes also undergo a change. In these latter organs the germ cells become pushed out to the periphery, so that the center of the embryonic testis is occupied only by stroma cells. This condition was reported also by Essenberg ('23) for *Xiphophorus*.

Shortly after the germ cells crowd out to the periphery, some of the stroma cells in the center of the testis cluster together to form a duct. This is the beginning of the sperm duct. The two characters taken together—peripheral location of germ cells and presence of sperm duct—are a certain indication of maleness. Both characters develop shortly after the birth of the animal.

*b. 9 to 12 mm. Early development of testis (fig. 22).* At this stage the two gonads have united and partly fused, though not so closely as they do in the female. It might be mentioned that the testis, even to maturity, shows in its contour its bilateral origin.

The sperm ducts have become better organized and now extend through the entire length of the testis. The peripherally located germ cells have undergone repeated division

and now are found, for the most part, in nests or groups (fig. 22). Within the nests there are, besides the spermatogonia, a few scattered stroma cells, probably derived from the stroma. The total number of sex cells in the animal here figured was 1187, showing how extensive has been the division of the cells (table 3).

Scattered between the nests of germ cells, and more especially in the center of the testes near the sperm ducts; are still some loose stroma cells. A careful examination of these cells failed to convince the author that any of these were transforming into sex cells. It will be recalled that in the female at this stage there was evidence of such transformation, and this fact led the author to expect the same condition in the male. Therefore both the stroma cells and the cells of the growing sperm ducts were examined repeatedly for signs of transformation. The crowded condition of the testis at this time, together with the very active mitosis going on among the spermatogonia, makes conditions far from ideal for securing definite, clear-cut results on this question. As far as the sperm ducts are concerned, the writer is quite sure that none of the cells composing these ducts change into sex cells at this stage. As for stroma cells, the question is more difficult to decide, but the author is of the opinion that they do not transform.

*c. 16 mm. Late juvenile testis* (fig. 23). At this stage the sperm duct has enlarged and branched. The branches extend out toward the periphery of the testis, so that individual sections have the appearance of possessing several unconnected ducts.

The germ cells are still chiefly peripheral in distribution, as in the preceding stage, but the nests, or acini, have enlarged considerably. Some appear to be pushing in toward the center of the testis, a few even reaching the sperm duct. The more central nests are derived from cells which have broken away from the peripheral nests and undergone repeated division to form new acini. The peripheral acini are all composed entirely of spermatogonia, whereas some of the more

centrally located consist of spermatocytes. Mitotic figures are extremely common.

There seems to be a great deal of degeneration of the sex cells at this stage. This degeneration is confined largely to a zone just within the layer of peripheral nests (fig. 23). The degeneration is so extensive as to raise the question whether any of the primordial sex cells really push through toward the center of the testis and continue their development to the definitive stage.

The author is convinced that this degeneration has no significance in the history of the germ cells. Degeneration of cells in the gonad seems to be a common occurrence in many animals. Butcher ('29) noticed it in the lamprey and offered crowding of cells and insufficient blood supply as a possible explanation. Duesberg ('18) also found the condition and says, "Degenerating cells are, as in other testicles and especially in invertebrates, by no means infrequent in *Fundulus*."

Essenberg ('23), in his study of *Xiphophorus*, is certain that the primordial germ cells do not continue their development to the definitive stage, though he is uncertain what does become of them. He finds that tubular branches from the sperm duct extend out toward the periphery of the testis and that these tubules divide up into cysts or acini. The peritoneal cells lining the acini then change into germ cells. Van Oordt ('25) agrees with Essenberg on the latter point. All the definitive sex cells of the male originate in this way, according to Essenberg.

As will be seen, my own observations do not support this theory of seminiferous tubules constricting off to form germ-cell lacini. In *Platyopocilus*—and observations on *Xiphophorus* convince me that the condition is the same in the two forms—there are no seminiferous tubules. The acini form from preexisting acini at the periphery of the testis, which in turn are descended from the primordial germ cells, and as they form are pushed in toward the center of the testis much as are the sex cords in the mammalian ovary. Neither the peritoneal covering of the testis, the stroma cells, nor the cells



of the sperm ducts showed any evidence of transforming into sex cells.

*d. 25 mm. Young mature male* (figs. 24 to 28). In the mature testis the two halves are fused throughout the length of the gland, but the originally paired condition is still easily discernible (fig. 24). The sperm ducts have enlarged considerably and are much branched. In the adult these sperm ducts seem always to contain spermatozeugmata or clumps of mature spermatozoa (figs. 24, 26). Each duct is lined with a single layer of cuboidal epithelial cells. Just caudad of the testis the two sperm ducts unite to form a short vas deferens. Outside the sperm ducts, in the body of the testis, are the acini or cysts of germ cells. Those nearest the ducts consist of almost ripe spermatozoa, while younger stages are located farther out. At the very periphery all the acini consist of spermatogonia, just as in the younger testis described in the preceding section (fig. 25). The entire testis is surrounded by a very thin peritoneal membrane.

Within a cyst the mature spermatozoa all lie with their heads pointed toward the outside, so that a cross-section shows a ring of heads within which the middle pieces and the tails lie projecting toward the center. Each cyst of spermatozoa is surrounded, at this stage, by a single layer of Sertoli cells (fig. 28). In a slightly earlier stage the heads of the spermatozoa are clumped about the Sertoli cells, so that in a cross-section through the center of a cyst the ring of heads appears broken up into about ten segments.

Since some interest has been shown in the interstitium testis of fishes, it seems well to describe this tissue as found in *Platypoecilus*. The description given by Van Oordt ('24) for *Xiphophorus* applies very well for *Platypoecilus* also. The acini at the periphery of the testis are crowded so closely against each other that there is very little room for an interstitium. Toward the center, however, and particularly between the lobes of the sperm ducts are areas of more abundant interstitial tissue which appear to contain all stages of cells from typical connective fibroblasts to large oval cells

of Leydig with compact round nuclei (fig. 27). The cells of Leydig are not numerous; the best examples are found toward the anterior end of the testis.

No evidence was found of transformation of soma cells into germ cells in the adult testis. In this respect male and female gonads are similar. The cells of the covering peritoneal membrane of the testis are very much flattened. None of them show any indication of becoming germ cells. Since spermatogonia are practically confined to the peripheral region and lie just beneath this membrane, it would seem that if there is any change of somatic into germ cells it would of necessity occur on the part of the thin peritoneal cells.

The only other cells that could possibly transform into spermatogonia lie within the body of the testis, away from the periphery. Such, for instance, are the interstitial cells and the epithelial cells lining the sperm ducts. If these transformed, one would find spermatogonia in the center of the testis. No spermatogonia are found in this position, so that one must conclude that in the adult testis no transformation of soma cells into sex cells occurs.

### *Discussion*

In the male *Platyopocilus* the definitive sex cells are derived from only one source: the primordial germ cells. Both Essenberg ('23) and Van Oordt ('24) claim that in *Xiphophorus* the definitive germ cells arise from the epithelial cells lining the tubules within the testis. From their very great similarity in other respects one would expect conditions to be the same in these two species. The author can only state that in *Platyopocilus* the evidence does not support the view that the definitive male sex cells come from any other source than the primordial germ cells.

In the immature testis of *Platyopocilus* entire acini disintegrate. Such disintegration has been reported in the case of several other organisms, and does not appear to be significant in the history of the germ cells as a whole. Certainly it does not occur to any extent great enough to destroy all

the reproductive cells descended from the primordial germ elements.

The history of the germ cells, then, is not the same in the female and the male *Platypocilus*. In the male there is no transformation of somatic cells into germ cells, while in the female such transformation does occur on a small scale. In both sexes the primordial sex cells give rise to definitive reproductive cells.

#### CONCLUSIONS

From a study of a series of ontogenetic stages of *Platypocilus maculatus* from a stage just preceding coelom formation, the following conclusions are drawn:

1. At a very early stage the primordial germ cells are found in the peripheral margin of the lateral mesoderm, from which layer they probably originate.

2. The primordial sex cells are capable of active amoeboid motion and wander mesiad until well embedded in the lateral mesoderm. Subsequent translocation is probably passive.

3. There is no transformation of somatic cells into germ cells in the indifferent gonad.

4. The earliest sign of sex differentiation occurs some time before birth and consists in a marked proliferation of germ cells in the female gonad.

5. The walls of the ovarian cavity (germinal epithelium) are formed out of the peritoneal covering of the gonad with an infiltration of cells from the stroma of the organ.

6. The walls of the sperm ducts are formed out of the stroma cells of the gland.

7. Definitive ova are derived from: 1) primordial germ cells, chiefly, and, 2) cells of the germinal epithelium, to a much less extent.

8. Definitive spermatozoa are derived entirely from primordial germ cells.

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## PLATES

All drawings were made with the aid of a camera lucida.

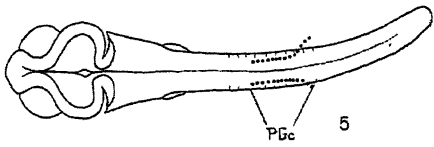
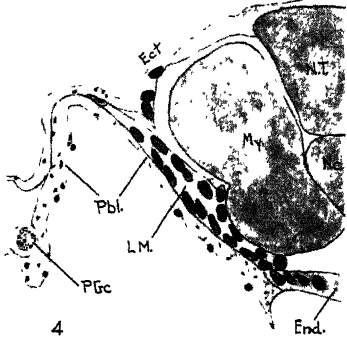
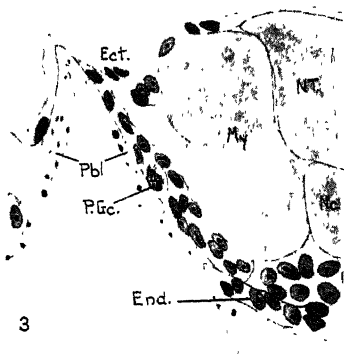
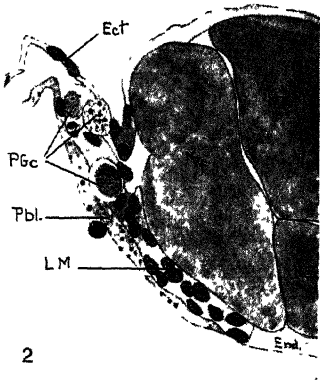
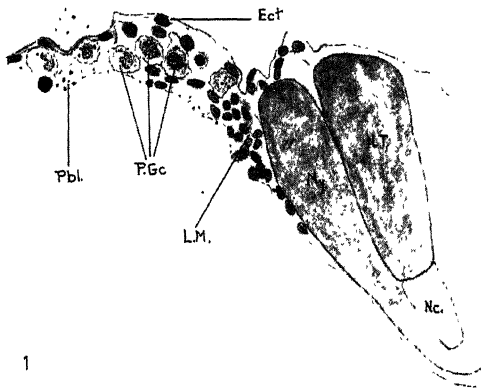
### ABBREVIATIONS

<i>Bl.C.</i> , blood cells	<i>Nc.</i> , notochord
<i>Bl.V.</i> , blood vessel	<i>N.O.</i> , nest of oocytes
<i>Co.</i> , coelom	<i>N.T.</i> , neural tube
<i>D.Gc.</i> , disintegrating germ cells	<i>O.</i> , ovum
<i>Do.</i> , dorsal aorta	<i>Oo.</i> , oogonia
<i>Ect.</i> , ectoderm	<i>Ov.C.</i> , ovarian cavity
<i>E.E.</i> , external epithelium	<i>P.</i> , peritoneum
<i>End.</i> , endoderm	<i>Pbl.</i> , periblast
<i>Fol.</i> , follicle cells	<i>P.Gc.</i> , primordial germ cell
<i>G.</i> , gonad	<i>Sp.D.</i> , sperm duct
<i>G.A.</i> , germ-gland anlage	<i>Spg.</i> , spermatogonia
<i>Gc.</i> , germ cells	<i>Spz.</i> , spermatozeugma
<i>G.E.</i> , germinal epithelium	<i>Srt.</i> , Sertoli cell
<i>Hd.</i> , heads of spermatozoa	<i>St.C.</i> , stroma cell
<i>Int.</i> , intestine	<i>Str.</i> , stroma
<i>Ley.</i> , cells of Leydig	<i>Tl.</i> , tails of spermatozoa
<i>L.M.</i> , lateral mesoderm	<i>Tr.O.</i> , newly transformed oogonium
<i>Mes.</i> , dorsal mesentery	<i>W.D.</i> , wolffian duct
<i>Meso.</i> , mesorchium	<i>Y.</i> , yolk
<i>My.</i> , myotome	

## PLATE 1

### EXPLANATION OF FIGURES

- 1 A portion of an embryo 1.6 mm. in length, passing through the germ-cell region. At this stage the germ cells are amoeboid in shape.
- 2 A portion of an embryo 1.2 mm. in length, passing through the middle of the germ-cell region. This embryo, though shorter, is more advanced than the preceding specimen.
- 3 Same embryo as figure 2, but through most cephalic part of germ-cell region.
- 4 Same embryo as figure 2, but through most caudal part of germ-cell region.
- 5 Dorsal view of 1.8-mm. embryo, showing distribution of germ cells.





## PLATE 2

### EXPLANATION OF FIGURES

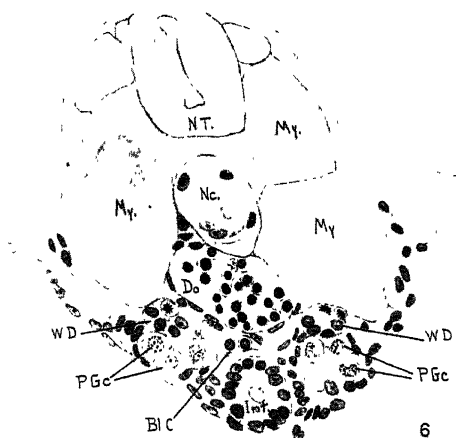
6 A portion of an embryo 1.8 mm. in length, through the center of the germ-cell region.

7 A portion of same embryo as figure 6, toward caudal end of germ-cell region.

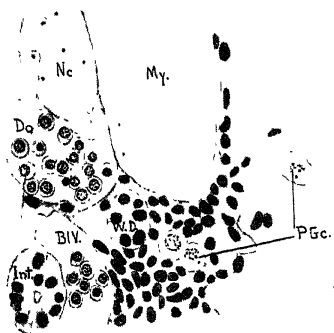
8 A portion of same embryo, still farther caudad. Note absence of germ cells from region below wolffian duct.

9 A portion of an embryo 2 mm. in length, through the germ-cell region.

10 A portion of an embryo 3.5 mm. in length, through the germ-cell region. Beginning of germ-gland anlagen. The anlage on the right contains no germ cells in this section.



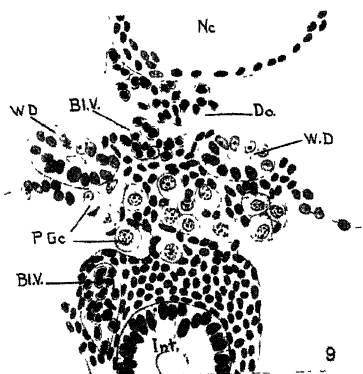
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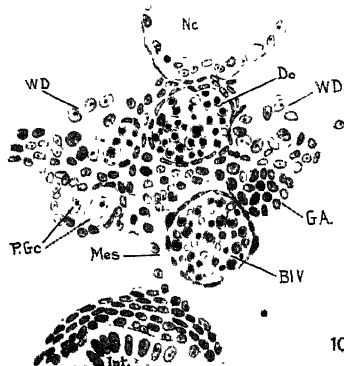
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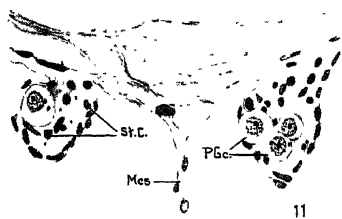


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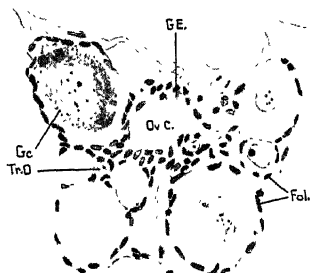
### PLATE 3

#### EXPLANATION OF FIGURES

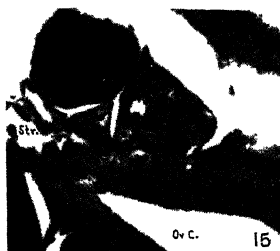
- 11 Section through the gonads of an embryo 5 mm. in length.
- 12 Section through the ovary of a fish 6.5 mm. in length. Ovarian cavity not yet formed.
- 13 Section through the ovary of a fish 8 mm. in length. Ovarian cavity and germinal epithelium formed.
- 14 Photomicrograph of a section through the ovary of a fish 24 mm. in length. Almost mature fish.
- 15 Photomicrograph of a portion of same section as in figure 14, showing nest of germ cells breaking up as a result of the growth of one of the cells.
- 16 Photomicrograph of a portion of same ovary, showing newly transformed germ cell in the germinal epithelium.
- 17 Drawing of the germ cell shown in figure 16.
- 18 Photomicrograph of a portion of same ovary, showing another newly transformed germ cell in the germinal epithelium.
- 19 Drawing of the germ cell shown in figure 18.
- 20 Photomicrograph of a portion of same ovary, showing neck of cells connecting large oocyte to the germinal epithelium. Within this neck is one newly transformed germ cell.
- 21 Photomicrograph of a section through a mature ovary. Ova fully formed and filled with yolk.



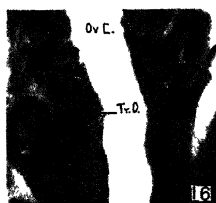
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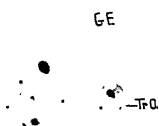
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## PLATE 4

### EXPLANATION OF FIGURES

22 Section through testis of a fish 9.5 mm. in length.

23 Section through left half of testis of a fish 16 mm. in length.

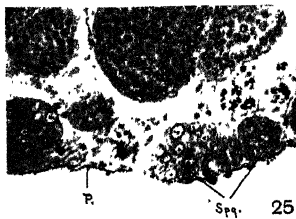
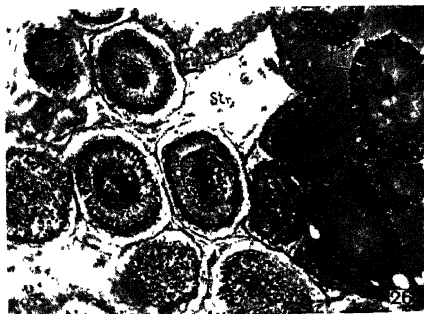
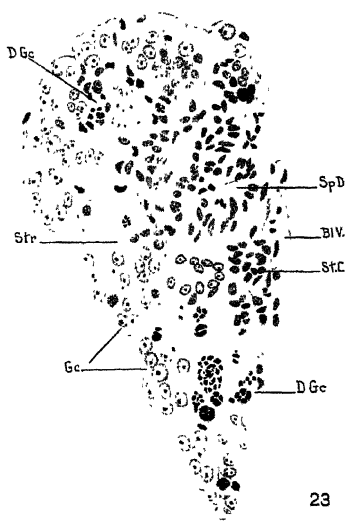
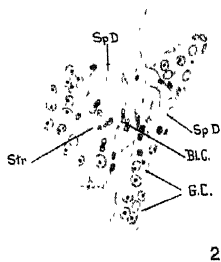
24 Photomicrograph of section through testis of a mature fish, 25 mm. in length. The lobed condition still gives evidence of the bilateral origin of the two halves. The edge of the testis somewhat out of focus. Note the acini, and the spermatozuigmata in the sperm ducts.

25 Photomicrograph of portion of outer edge of section through same testis. Outer acini contain spermatogonia; inner, spermatocytes.

26 Photomicrograph of portion of same testis, showing part of a sperm duct containing spermatozuigmata. Acini 1, 2, and 3 show various stages in the process of an acinus breaking out of the body of the testis into the sperm duct.

27 Photomicrograph of portion of same testis, showing interstitial tissue and a cell of Leydig.

28 Photomicrograph of a single acinus of spermatozoa, showing layer of Sertoli cells. Heads of spermatozoa somewhat out of focus.





# SOME HISTOLOGICAL EFFECTS OF ULTRASONIC WAVES ON CELLS AND TISSUES OF THE FISH *LEBISTES RETICULATUS* AND ON THE LARVA OF *RANA SYLVATICA*

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## THREE FIGURES

### AUTHORS' ABSTRACT

Some pathological effects of supersonic waves produced by the piezo-electric oscillator described by Wood and Loomis ('27) are described.

Death of fish and frogs was due to haemolysis of erythrocytes within the gill capillaries. Disruption of gill filaments, trauma, and extravasation in the head and gill region and in the peripheral musculature were noted.

No effect on the nervous system was apparent histologically.

Organisms were not killed in water under hyper- or hyponormal pressures. In these cases no cavitation of gases took place.

Isolated frog sartorius muscle in Ringer's was not damaged.

Bubbles of gas were easily observed within the muscle cells of the tadpoles when such cells were not more violently disturbed. Such bubbles were abundant in the more fluid muscles of four- or five-day tadpoles, less abundant in 15- to 35-mm. *Lebistes*, and entirely lacking in adult frog *sartorius*. Protoplasmic viscosity differences probably explain the gradient.

The above-observed intracellular cavitation explains the muscular destruction, the haemolysis, the change in specific gravity of organisms subjected to the wave treatment, and may offer light upon the stimulatory effect of such waves on excitable tissues (compare Harvey, '28).

The destructive effect of high-frequency sound waves of great intensity acting upon living organisms was first noted by Langevin at Toulon in 1917, while experimenting with a device for locating submarines by the echo of a narrow beam of ultrasonic waves. Fish in the path of the beam were reported to have been killed. Wood and Loomis ('27) corroborated this observation and added to it other cases, such as the killing of frogs by relatively short exposure to the sound waves. The cause of the death of the organisms was not determined in either instance.

A number of studies have appeared subsequent to the two earlier ones mentioned. Wood and Loomis ('27) and Harvey and Loomis ('28) have reported the laking of frog blood corpuscles, the disruption of the cell membranes of *Elodea* and



*Spirogyra*, and the tearing of *Paramoecia* by treatment with the supersound waves. Harvey and Loomis produced interesting variations of these effects through varying the intensity of the irradiation. Schmitt, Olson, and Johnson ('28) and Schmitt ('29) led the waves to cells through microneedles. Harvey, Harvey, and Loomis ('28) studied the effect of the waves on *Amoeba*, *Arbacia* eggs, *Arbacia pluteii*, *Fundulus* eggs, etc., and found mechanical disturbances such as stirring of the cell contents and indention of the cell wall most prominent. They failed to note any stimulating effect on the heart of *Fundulus* embryos or on the gill cilia of *Mytilus*. Harvey and Loomis ('29) reported the breaking up and killing of luminous bacteria when exposed to sound waves of approximately 400,000 frequency.

More recently, it has been shown by Johnson ('29) that hemolysis of corpuscles does not occur in liquids containing no dissolved gases. It has also been shown that increased pressures prevent the destructive effect on cells and also prevent the formation of gas bubbles in the medium by cavitation.

Harvey's ('29) study on the effect of high-frequency sound waves on heart muscle and other irritable tissues demonstrated that irritable tissues may be stimulated slightly by the waves. Heart muscle responded by an increase in rate, but decrease in amplitude. Nerve-muscle preparations could be stimulated to contraction through the nerve. Smooth muscle showed evidence of increased tone on exposure.

The present experiments were undertaken with a view to determining the cause of death in multicellular organisms subjected to treatment with ultrasonic waves. As materials the young of the fish *Lebistes reticulatus*, ranging from 15 to 30 mm. in length, and the tadpole of the frog, *Rana sylvatica*, at an age of four or five days, were employed. The source of the supersonic radiations is described fully by Wood and Loomis ('27). The apparatus used in the first experiments consisted essentially of a piezo-electric oscillator of quartz operated at 50,000 volts and with a frequency of 300,000. It

is in the laboratory of A. L. Loomis at Tuxedo Park, New York. Later experiments were performed in the physiological laboratory at Princeton, using an apparatus of the same type, but employing less power.

Several specimens of *Lebistes* were taken to the Loomis laboratory and subjected to the high-frequency irradiation. The fish selected were of two orders of size, one group being about 15 mm. and the other about 35 mm. in length. One large and one small fish were exposed together, the length of the exposures being thirty, forty-five, and fifty-five minutes, respectively, for the three pairs. The animals were placed in a large test-tube cooled by a coil of cold water to keep down external and internal heating effects as much as possible. A stream of air was bubbled through the medium to insure against suffocation, which might follow driving of the dissolved gases out of solution by cavitation. Under these precautions the fish could not be pronounced certainly dead for twenty to twenty-five minutes. The smaller ones seemed to succumb in less time than the larger. The first reaction was a violent agitation which lasted but a few seconds, after which a gradual subsidence of activity ensued, passing finally into complete lethargy and death. The behavior was not unlike what would be expected in cases of suffocation through deprivation of oxygen, except for the first spasms. During the early part of the exposure bubbles of gas were seen to collect on the body surfaces, due to cavitation of dissolved gases.

After exposure each pair of fish was allowed to remain in the water for a half hour to observe any possible recovery, and then plunged in Bouin's fixative. Complete serial, longitudinal sections of the fish were prepared after celloidin-paraffin embedding. Staining was with Delafield's haematoxylin with a counterstain of eosin. Control animals, killed by immersion in Bouin's fluid, were also sectioned and stained for comparison.

Direct evidence that the fish had died from some destructive effect of the sound waves was sought in the vascular, respira-

tory, and nervous systems. Death of the animals was undoubtedly due to suffocation incumbent upon disruption of the fine capillaries of the gill filaments and hemolysis of erythrocytes. The filaments bearing the respiratory membrane were quite noticeably distorted, and in some cases, after long exposure to the raying, the membrane itself was ruptured.

The hemolysis of red corpuscles seems to have taken place largely, if not entirely, in the blood vessels of the gills. Calculations based on careful counts of corpuscles in various parts of the body showed the following condition (in 12-mm. fish):

ANIMAL	IN SYSTEMIC VESSELS			IN GILLS		
	Counted	Hemolyzed	Per cent	Counted	Hemolyzed	Per cent
I (30 minutes)	500	183	35.6	200	91	45.5
II (45 minutes)	500	171	34.2	200	147	73.5
III (55 minutes)	500	177	35.4	200	196	98.0
IV (Control)	500	31	6.2	200	12	6.0

The per cent of hemolyzed corpuscles outside the gills is seen to have been approximately the same in all three experimental animals. On the other hand, the per cent of hemolyzed cells in the gills rose steadily with the exposure time. This would indicate that hemolysis was largely localized in the gills. When 35 per cent (circa) of destruction was accomplished, the flow of blood stopped and subsequent hemolysis affected only the corpuscles which remained in the gills. The data would also indicate that the circulation of blood ceased after somewhat less than thirty minutes of exposure.

Other parts of the vascular system showed an occasional rupture of a small blood vessel, usually peripheral in position, indicated by more or less extravasation. The cardiac muscle seemed entirely undisturbed.

The nervous system showed no result of the irradiation that can be described as general. One observer (Professor Dahlgren) suggested that the cells seemed to have been shaken and the nuclear contents disturbed, but no specific abnormalities could be discerned.

While the cardiac muscle seemed free from injury, the most apparent damage to tissues was found in the skeletal musculature, particularly in muscles close to the surface of the body. The usual type of striation present in fish muscles after Bouin fixation was altered, in fibers that were not more violently disturbed, to a type that may be described as accompanying extreme relaxation. The O stripes assumed an unusual prominence, while the Z stripe was observable only with difficulty under the highest available powers of the microscope. Such an effect has been seen to accompany suffocation, and in the light of evidence cited above, may be so interpreted here. The relaxed appearance was general throughout the entire musculature.

Muscle fibers over the surface of the body, and to varying depths up to 0.25 mm., were disorganized strikingly. All trace of fibrillar structure and striation was destroyed, and in many cases, the contractile material and sarcoplasm were thrown into homogeneous masses at roughly regular intervals within an otherwise empty sarcolemma. Irregularly disposed nuclei were visible in some of the nodes, but they most frequently remained, apparently attached, in the sarcolemma. A few long fibers showed as many as six nodes separated by internodes of 90 to 100  $\mu$ . In the segmental muscles the most frequent position of the node was at the contact of the fiber with the connective-tissue wall between myotomes. In the more peripheral fibers even the sarcolemma was destroyed. Between this extreme condition of disruption and the abnormally relaxed condition previously noted, there were visible all intervening gradations. This effect was chiefly peripheral, but was also obvious at points of rigid connection, particularly in the head and gill region. The depth of the destruction, measured from the body surface, varied as the exposure time.

Other experiments similar to the first set were carried out, this time in such a way that there could be no possibility of "seeing what the histologist expects to see." The fish were rayed at Tuxedo by Professor Harvey. The observer was not

informed which lot was treated and which was the control. The treated fish were exposed only three minutes and were not dead when fixed. No difficulty was experienced in selecting the treated fish, although hemolysis and destruction of muscular tissue were much less marked than in specimens exposed for a longer time. The peculiar appearance of the muscle striations was found to be an infallible guide.

In another experiment some specimens of *Lebistes* were exposed to the waves for four minutes in water from which dissolved gases had been evacuated previously. Previously published results on the effect of the sound waves upon single cells in evacuated media led to the expectation that no hemolysis or tissue destruction would result. The fish came to the surface before treatment was begun and seemed to be killed by the lack of oxygen. They did not show the characteristic agitation at the beginning of the irradiation. The temperature of the water rose to 39°C. during the treatment, but this was shown by later experiment to have no fatal effect on the organisms. A second lot of fish was treated two minutes in normal water. The characteristic primary spasms and gradual subsidence of activity were observed. In all cases the fish come to the surface during treatment and remain there if the exposure has been long enough to disable them. A third set of controls were prepared. The identity of the three lots was again withheld from the operator, but no difficulty was experienced in selecting the subjects of the three types of treatment. Those fish rayed in evacuated water showed no effect except the peculiar striation type in the muscle fibers, which has been associated with death by suffocation. Thus there was a corroboration of the observation that supersonic waves are not destructive to cells in evacuated media. The second lot of treated fish show pathological conditions identical with those described in other experiments.

A specimen of *Lebistes* subjected to the supersonic waves while under increased pressure (about 15 pounds) was in no way affected. There was no agitation, nor was there any swimming to the surface as under normal pressures. The

pressure applied was sufficient to prevent release of dissolved gases from the medium by cavitation.

Doubts of the existence of muscular destruction in the treated fish led to further experimentation to determine how much of the effect was artifact.

Isolated sartorius muscles of a frog were treated for five minutes in Ringer's solution, one under normal pressure and the other under diminished pressure with dissolved gases evacuated from the medium. Histological study revealed no effect whatsoever in either case.

Tadpoles of an age of four to five days were next exposed to the ultrasound waves under normal conditions of pressure and gas solution. The results were of an order entirely unexpected. At the onset of exposure the tadpoles were violently agitated, swimming recklessly about, but tending toward the surface. Very shortly, they relapsed into complete lethargy at the surface of the water. An exudate was given off the nature of which was not determined. At one minute there were no signs of life; at two minutes the organisms were fixed in Bouin's. Sectioning and staining were the same as with the fish of earlier experiments.

There was very little hemolysis of blood cells, although a small amount was observable in the gills. Extravasation was more general than in the fish. The heart seemed unaffected.

The segmental muscles appeared as though they had been subjected to some digestion process, but upon close examination revealed an orderly progress of the destructive process. In figures 1 and 2 are shown photomicrographs of segmental muscles in the treated specimens. Figure 3 is from an untreated control for comparison. Destruction of the fibers proceeded from the surface inward and all stages of the process are observable in a single segment. The more external fibers are completely disrupted, but the deeper fibers are less affected. Within them, distinctly intracellular, are to be seen bubbles of gases apparently released from solution in the fluids of the cell. It is these bubbles which by their multiplication and aggregation have caused the disruption of

the cells. That the bubbles are within the cells is easily demonstrated in sections by focusing above and below the spherical spaces. Cross striations are easily seen. They are well shown in the photomicrographs.

Here, then, is not only corroboration of the results obtained from histological study of *Lebistes*, but also an explanation of the peculiar pathological conditions observed after supersonic treatment. There remains to be explained the great disparity in amount of destruction in the tadpole and in the fish, as well as the failure to produce such an effect in living but isolated sartorius muscles. We can only suggest that if the destruction is due to release of gases from solution within the cell, as seems indicated, the relative amounts of fluids in the three types of cells may have something to do with the discrepancy. It is true that the segmental muscles of the young tadpoles were still in process of differentiation, fluid constituents making up a large part of their bulk; the muscle cells of the young fish were much more compactly filled with differentiated fibrillae, leaving a relatively small volume of fluid; while the adult muscle cells of the frog contain, relatively, a very insignificant amount of fluid in which dissolved gases might be contained.

The appearance of the bubbles of gas in the cells explains the behavior of the fish and tadpoles in swimming to the surface of a medium during exposure. It is easily conceivable that release of gases from solution, in such volume as is revealed by the accompanying photographs, might lower the specific gravity of the entire animal substantially.

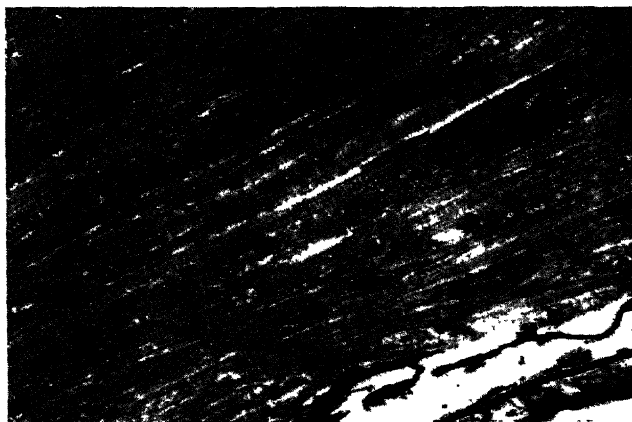
Fig. 1 Photomicrograph of segmental muscle of tadpole exposed two minutes to supersonic waves. Large bubbles of gas are easily discernible in the innermost fibers. Destruction is more complete peripherally. Section longitudinal, 10  $\mu$  in thickness. Fixed in Bouin's fluid, stained with Delafield's haematoxylin and eosin.  $\times 450$ .

Fig. 2 Photomicrograph as above except in different region and  $\times 400$ . Large numbers of small intracellular gas bubbles are shown. In many cases the striations outside the bubble may be seen. Destruction of the more peripheral fibers is typical in this section.

Fig. 3 Photomicrograph of segmental muscle of control. Treatment was exactly the same as in figures 1 and 2 except for the exposure to supersonic waves.  $\times 450$ .



2



Figures 1 to 3



The absence of agitation during treatment under increased pressure is also explainable. Cavitation is prevented not only in the medium, but within the cells; hence there is no sensible effect within the fish.

Release of gases from solution by cavitation has been observed to be a constant effect of ultrasonic waves (Wood and Loomis, '27). This result is due to the rapid alternation of high and low pressures in local areas. As advocated by Johnson ('29), it seems likely that such a simple physical phenomenon may offer an explanation for many observable effects of the waves on living organisms whose tissues are composed largely of fluids in which gases are dissolved.

#### SUMMARY

1. It is shown that blood cells of *Lebistes* are hemolyzed in the gills during exposure to supersonic waves. Death is probably due to suffocation incident upon such hemolysis.

2. Destructive effects of supersonic waves on skeletal muscles of *Lebistes* and the tadpole are described. They are to be explained as resulting from release of gases from fluid constituents of the cells by intracellular cavitation. Spaces left by the gas bubbles can be observed in the fixed tissue.

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# THERMAL RELATIONSHIPS IN THE NEUTRAL-RED REACTION

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## AUTHOR'S ABSTRACT

Neutral-red staining in *Daphnia*, legume seeds, starfish eggs, and *Palaemonetes* shows reactions to temperature changes which may be correlated with the thermal reactions of enzymes.

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## INTRODUCTION

According to the recent review of Waldschmidt-Leitz ('29), physiologists are in agreement that the process of digesting proteins occurs through the intervention of a hydrolytic protease. Similarly, the synthesis of proteins is brought about by a dehydrating protease. Bayliss ('23) and others have found that these two processes are activated, not by two distinct enzymes, but by one and the same enzyme, acting in the one case in a water-rich medium and in the other in a water-poor medium.

The fluids in which proteolytic digestion is known to occur have been found to bring about a high local concentration of the azine dye neutral red from very dilute solutions (Koehring, '30). These fluids include those secreted into the

stomachs of metazoan forms having extracellular digestion and also the digestive vacuolar fluids of all phagocytic cells and of protozoa. Now there is biochemical evidence of a relationship between enzymes and azine dyes. First, Robertson ('07), Holzberg ('13), Marston ('23), and Robertson ('28) have studied the precipitation of a number of proteolytic enzymes by azine dyes (neutral red, Janus green, safranin, etc.), determining that the colored precipitates remain highly active proteolytically and that they are of the nature of chemical compounds. Secondly, Epstein and Rosenthal ('24) determined that azine dyes (neutral red and safranin) combine with trypsin, experimentally making use of this fact both in in-vivo and in-vitro reactions. Thirdly, Eblinger and Funk ('27) used safranin to precipitate and purify pepsin, the precipitate being a highly proteolytic compound. Fourthly, Richardson and Cannon ('29) have reinvestigated the whole subject of the reaction of azine compounds with proteolytic enzymes, using pepsin and trypsin with neutral red and safranin.

The suggestion was made by Koehring ('30) that the striking accumulation of neutral red in the fluids in which proteolytic digestion occurs in vivo is brought about by the combination of the azine radical with the enzyme. Since the enzyme itself is abundant and is conserved within the lumen, each dye molecule that enters is retained and the final color is an index of the concentration of the enzyme wherever it is active. No specificity for proteolytic enzymes and azine dyes is implied (save by Robertson, '28, and also not denied by Richardson and Cannon, '29); it is probably just a fortunate coincidence that one of the substances precipitating without inactivating these enzymes is non-toxic to cells when used in such a manner as not to stimulate, save to a very slight degree, excessive enzyme secretion, thereby rendering visible within living cells the operation of physiological functions and the structures essential to these functions.

On this supposition that local concentration of neutral red indicates the site of an active enzyme, whether in hydrolytic

or synthetic phase, examinations have been made of a wide variety of cells. It is found that besides the concentrations in watery lumina, presumably hydrolytic, there is often concentration on certain cellular bodies of lipoidal nature, either chondriosomes or chondriosome derivatives; and a further conclusion is drawn that these bodies are presumably the site of the synthetic activity of the enzymes.

It is well known that all animal cells possess structures that will stain with azine dyes at certain periods in their life history and not at all or only with strong solutions at other times (Lewis and McCoy, '22; Sabin, Cunningham, and Doan, '25; Parat, '28; Hibbard, '28; Nath, '28, etc.). The same situation holds for plant cells (Guilliermond, '29), since all plant cells have stainable structures at certain periods in their life cycles. Animal and plant cytologists agree that the structure most usually and prominently staining with neutral red is of vacuolar nature. Guilliermond's ('29) summary holds for both plant and animal cells: "The vacuome problem is definitely solved as to morphology but it should be investigated as to physico-chemistry on which we now depend for a better knowledge of cell physiology." Physiologically, vital staining has had little significance. Some cytologists, interested in the vital properties of cytoplasmic inclusions, cautiously attach physiological import to the morphological conditions their technique has brought out. The whole situation with respect to azine vital staining shows not only unstandardized technique, but a failure to approach the problem from a physiological standpoint.

Koehring ('30) has perhaps advanced a little further in an investigation of the subject as a whole and the proffered suggestions offer two definite directions for progress. First, the technique of vital staining is to be regulated by the use of unstained controls as well as by different degrees of staining. Only material is suitable for study in which continuous staining during periods of growth, development, or some other well-recognized type of change can be compared with these normal changes in unstained controls. On no other

basis can a physiological constancy in vital staining be assured. Secondly, in proposing the conception of a combination with active enzymes as the underlying principle of azine vital staining, an opening is created for investigations guided specifically by what is known regarding enzyme action. Can vital staining phenomena be experimentally correlated with enzyme phenomena? One such avenue of approach is followed in this paper.

#### THE PROBLEM STATED

Every form of animal life is more or less delicately adjusted to temperature conditions, and optimum temperatures are somewhat specific. Sometimes within a genus different species show very different optimum temperatures for growth, development, and reproduction. However, optimums for enzymes, known to be temperature-dependent, do not correspond to the most favorable temperatures for the organisms which produce them. In fact, the thermal properties of enzymes are rather universal, so that similar enzymes, e.g., proteases, from all sources, cold-blooded and warm-blooded animals, bacteria and protozoa, green plants and saprophytes, show about the same temperature points.

These temperature points for proteases in general are roughly as follows:

1. At 0°C. and below the enzymes are not injured.
2. As the temperature is increased the velocity of the enzyme reaction is accelerated two or three times for every 10°. Up to the optimum temperature for the animal increases in temperature bring about similar increases in enzyme activity and in other physiological processes, so that in this range these processes keep in step fairly well.
3. An optimum is reached for hydrolyzing enzymes around 38° to 40°C., far above the optimum temperatures for most organisms.
4. Further increase of temperature rapidly diminishes the reaction velocity until reactions become irreversible, cease, and the enzyme is completely inactivated or 'killed.' The

inactivating temperature usually is between 60° and 70°C. for enzymes in solution. There is some difficulty in determining definitely the destruction temperatures, since they vary with the duration of heat and the hydrogen-ion concentration. Always, however, destruction is at a temperature much higher than that of the most favorable temperature for hydrolysis in the organism.

If physiological neutral-red staining is an index of the site and intensity of normal enzyme activity in the organism, such staining may show differences in response to differences in temperature—differences which can be correlated with these thermal properties of enzymes.

#### MATERIALS USED

The materials used for these investigations are:

*Daphnia pulex*, a minute crustacean in which the neutral-red complex of every cell in the body as well as the digestive tract enzymes may be demonstrated in cultures living in dilute solutions of neutral red (Koehring, '30). The cellular reactions of the whole organism to temperature changes may be observed under the microscope.

Leguminous seeds, which may be grown to large seedlings with brilliantly stained aleurone cells showing all stages of protein hydrolysis. Reactions to temperature are observed both in vivo and in vitro.

Starfish eggs, treated both vitally and supravitaly with neutral red, in relation to their artificial parthenogenesis induced by momentary raising of the temperature.

*Palaemonetes*, a large, transparent crustacean in which the stomach is visible brilliantly stained in animals submitted to dilute neutral-red solutions, and with also some staining of the carapace and gills macroscopically visible. The neutral-red reactions in this material are on a large scale and thus indicate macroscopic in-vivo reactions comparable to test-tube reactions of neutral red plus enzymes.

#### TEMPERATURE RELATIONSHIPS IN THE STAINING OF DAPHNIA

1. If a stained *Daphnia* is put in a hanging drop between a no. 0 cover-slip and a depression slide and the slide is held against the heavily frosted side of a vessel containing freezing mixture, the cover-slip becomes frozen to the slide. The drop of water surrounding the *Daphnia* approximates zero,

but is not frozen. Examination of this preparation under the microscope shows perfect staining and normal muscular action and heart beat. If the preparation is reversed, with the thin cover-slip against the frosted surface, the *Daphnia* becomes encased in ice crystals. When such a preparation that has remained frozen for one minute or for one hour is placed upon the microscope, the rapid movement of melting crystals at first interferes with observation. By the time the organism becomes clearly visible, the anterior intestine is losing the pink color in the lumen (discussion of this point later), and distortion of the whole body is taking place. All cellular neutral-red bodies, however, are intact and of the same brilliancy as before freezing. They may remain in this condition, at ordinary room temperatures, for more than an hour.

The animal is dead and distorted; presumably the cells are dead. Nevertheless, the neutral-red stain in combination with the vacuolar fluids remains unchanged. It is well known that freezing an animal in full physiological activity will kill the animal, but not the enzymes.

2. *Daphnia* stain very slowly at 1° to 2°C. After twelve hours in a 1/1,000,000 solution of neutral red, only the intestinal lumen, the phagocytes, and the ovary are stained delicately. At this temperature all body movements as well as the heart beat are slow, although vigorous.

*Daphnia* are most active, flourish best, and stain most brilliantly and completely at temperatures slightly lower than 20°C. At such temperatures, e.g., at 16°C., the animal is so susceptible to the stain that a 1/1,000,000 solution of neutral red is toxic. Complete staining involving the demonstration of the neutral-red bodies of every cell in the organism is accomplished by a 1/2,000,000 dilution, but this is slightly toxic in that reproduction is somewhat interfered with. In the extreme dilution of 1/5,000,000 there is also complete staining, with every manifestation of normal appearance, activity, and reproduction.

Brown's ('29) observation on the optimum temperature for *Daphnia* states that "Daphnia pulex flourishes best at temperatures of 20°C. and below, and survives with difficulty at 27°C." At temperatures above 20°C. the above-mentioned dilutions of neutral red fail to stain *Daphnia* as they do at lower temperatures. At 25° to 26°C. the 1/5,000,000 dilution never stains more than the phagocytic and gonad systems and these faintly and briefly; the 1/2,000,000 cannot be used as a complete stain, while the 1/1,000,000 is not at all toxic and will not stain the brain and carapace cells save fleetingly and extremely delicately. Even the muscle neutral-red bodies are usually so delicately stained that they may be detected only under high powers of the microscope.

The above variability in the effect of the stain seems definitely connected with the effect of temperature as it changes the whole metabolism of the organism. Sustained high temperature is a depressant to *Daphnia*; whether it is directly or indirectly an enzyme depressant cannot be stated. Just how the neutral red is handled by the organism under these conditions is not understood. It is taken up quickly, but never becomes brilliantly concentrated in the vacuoles, particularly in those cells which stain last in the progress of selective staining in the organism (see Koehring, '30, for description of this process). The whole intricate problem of decrease in tonicity at high temperatures is presented afresh, with one more puzzling factor to be accounted for.

When a culture reared at 19°C. is stained at 25°C., however, the effect is very different—brilliant staining of the intestinal lumen, phagocytes, ovary, and muscles may be accomplished within half an hour, a remarkably short time. A sudden but not prolonged rise in temperature, even above the optimum for the organism, is a marked stimulant for vital staining and so may agree perfectly with the enzyme parallelism.

3. The next step in this investigation involves killing the organism, for the optimum of proteolytic enzymes is around 40°C. and in-situ examination of enzymes at these tempera-



tures must of necessity be made on dead animals. Brown and Crozier ('28) report the lethal temperatures for *Daphnia pulex* for one-minute exposures as from 32° to 37°C. In the following experiments it is found that the lethal temperatures for one-minute exposures are 39° and 40°C.

When a stained *Daphnia* is pipetted into a beaker of water at these temperatures, all swimming movements are immediately inhibited, although intestinal, eye, jaw, and heart movements may not be stopped. A rigid criterion for whether recovery or death will eventuate from this partially narcotic state is found in the condition of the anterior curve of the intestine and in the lumina of the digestive coeca, which are also anterior. There are extracellular enzymes in these regions of the digestive tract, and in a stained animal the lumina are normally pink. If, after exposure to heat, these regions remain undistorted and the pink color is retained in the lumina, recovery is assured. If marked swelling has occurred and the color has faded out during the minute of exposure or in the first few minutes subsequently upon the microscope stage, the animal will not survive even though the heart and all muscular movements may become normal for a considerable time after cooling.

At these temperatures, even with recovery, as well as at somewhat lower temperatures which are depressant but not lethal, there is always some swelling in the anterior gut, the dilated lumen reaching a maximum width just ventral to the heart. This part of the body is far more sensitive than any other region under a variety of experimental conditions. Even under abnormal conditions the lumen fluids retain the pink of neutral red in varying degrees of intensity. Under markedly abnormal conditions involving injury to the lining cells, however, the stain always disappears, usually irreversibly. This part of the neutral-red system of this organism—the only extracellular staining—must be considered as showing a special reaction until more information regarding this very significant mechanism is obtained. (More facts on this subject will be presented in a forthcoming paper on the influence of narcotics on neutral red.)

At 39° to 40°C., whether the organism is killed or not, there is no marked change in any part of the cellular neutral-red system of the body. No cellular vacuoles lose color. Higher temperatures, 41° to 60°C., quickly killing the animal, exhibit the same phenomenon—no destruction of any cellular neutral-red combination taking place. Degrees of intensification of color are difficult to distinguish in small, already brilliant bodies, but enough such change is often definitely noted to warrant the statement that the neutral-red combination in the vacuoles is in some way heightened, especially between 40° and 50°. Occasionally a definite precipitate is thrown down in the large vacuoles of the shell-gland cells, the phagocytes, and the ovarian supporting tissue, the precipitate being so densely red as to appear almost black against the paler pink of the vacuolar fluid. Neutral-red bodies in these dead animals may be retained for many hours; within twelve to twenty-four hours they disappear.

A word may be said regarding current conceptions of the behavior of neutral red in the death of the cell. Cowdry ('24), Lewis and McCoy ('22), Bowen ('28), and numerous other investigators hold that the disappearance of neutral-red staining is a criterion for the death of the cell and that when color remains in the cell it is alive. Under certain conditions this is true; under a variety of other conditions it is not true, and the writer proposes to point out, in a later paper, exactly what these conditions are and how closely they parallel the phenomena of enzyme reactions under the same conditions. It is sufficient to say here that under one condition (death under exposure to temperature in a range known to heighten enzyme activity to its optimum) neutral-red color does not disappear from the vacuoles of the killed cells, and may even be intensified in these vacuoles.

4. By increasing the temperature, with one-minute exposures, a definite temperature is reached at which all staining is destroyed. For different individuals this point falls within a narrow range of 2° or 3°. For example, in a very flourishing culture an animal killed at 66°C. shows the preservation

of perfect cellular neutral-red staining; one killed at 68°C. loses all stain. This small range is characteristic for the entire culture; animals killed at 66°C. will remain stained and those killed at 68°C. lose their stain. Some animals killed at 67° retain the stain; others lose it. At some point between these two degrees something happens to release the stain from the combination which has been holding it firmly. Death and distortion, involving the physical and chemical changes in protoplasm known to occur in heat death, do not in themselves affect the neutral-red combination if the heat has not been greater than a certain critical temperature.

One culture consistently showed this change between 62° and 64°C. Another culture had a definite range lying between 63° and 65°C. In a culture recuperating from formalin poisoning the range lay between 59° and 61°C. No staining has ever disappeared before 59° and no staining has ever persisted above 70°C. in these experiments (290 animals were killed and examined individually in these ranges). Evidence points to the fact that cultures living and stained under optimum conditions have the highest destruction temperature for staining. Is one justified in suggesting that this is also the destruction temperature for their enzymes?

There is abundant evidence that stain in the ovary is more resistant to heat than in any other portion of the body. There are always animals killed within the critical range for stain destruction that still show color in the ovary. Now it is always true that the ovaries of *Daphnia* respond with a different color reaction—their neutral-red bodies are more acid than those in any other tissue in the body, so that the ovaries usually present a pink or bright rose appearance, in contrast with the more red to brick-red color of other cells of the body. Does not this difference in staining reaction, as well as the difference in heat resistance, indicate a vacuolar combining substance of different nature than in the other tissues? Is one justified in suggesting that this may be an enzyme difference, some specifically adjusted protease?

### *Summary*

There are four more or less definite ways in which enzymes and enzyme activity respond to temperature. Response to temperature of neutral-red staining in *Daphnia* falls into these four groups closely paralleling the enzyme phenomena. So far, enzyme reactions and neutral-red-staining reactions may be correlated.

#### TEMPERATURE RELATIONSHIPS IN THE STAINING OF ALEURONE CELLS

##### *1. Comparison of neutral-red staining in animal and plant cells*

In animal cells the vacuoles are in most cases watery, e.g., in *Amoeba*, *Paramecium*, and most tissue cells (Owens and Bensley, '29; Parat, '28, etc.). In this condition, at least in the Protozoa and in amoeboid digestive cells in Coelenterata, Platyhelminthes, Rotifera, and in vertebrate leucocytes and Kupffer cells, digestion observed within these structures indicates that there is active hydrolysis in the presence of water.

Cellular vacuoles need not remain hydrolytic if they become dehydrated, as happens in the development of the spermatozoa of almost all animals (Bowen, '22, and many other papers on spermatogenesis). The cytological evidence is that in such vacuoles a granule is slowly laid down during the dehydration of the vacuolar system to form the acrosome (Voïnov, '27; Champy, '23; Poisson, '27). That such a condensation or dehydration product may again become hydrolytically active in fertilization is suggested by Champy ('23) and by Hibbard ('28).

A close parallel to this phenomenon of a cellular vacuole system being at one time synthetic and at another hydrolytic is known to exist in the aleurone cells of seeds. In fact, it is one of the few cases in plant cells where the function of the vacuolar system is definitely known. The development of the aleurone cells is described by Mottier ('21). Protein, in the form of crystals in *Ricinus* or amorphous substances in legumes, is laid down in vacuoles. Dangeard ('23) and Guil-

liermond ('26, '29) have proved the vacuolar nature of these protein inclusions and their affinity for neutral red during both the stages of dehydration and hydration.

It is also a common laboratory experiment that while the nucleus, the cytoplasm, chloroplasts, leucoplasts, and all other constituents of the cell are only partially dissolved in gastric juice, the aleurone grains are readily and completely dissolved, thus indicating their protein nature (Palladin, 1923 edition).

Solubility of protein granules is demonstrated in vivo when seeds germinate; the vacuoles enlarge and the protein deposits gradually disappear. Just as the deposition of proteins, thought to be insoluble to vacuolar membranes, must have taken place by a synthesizing of protein precursors within each vacuole at the time it was formed in the ripening of the seed, so must disappearance of the proteins take place by hydrolysis when the vacuole is being hydrated during the water absorption of germination.

Now in resting seeds no active proteolytic enzymes are found (Buthewitsch, '01). In early germination these enzymes appear, gradually transforming the protein stores into asparagin and other amino substances. In the legumes, at least, the aleurone layer, as a definite protein store, is the site of this process in early hydration. If the neutral-red staining on the surfaces of the protein substance of aleurone cell vacuoles should be an indication of active enzymes upon these surfaces, then there would be such, 1) staining in living cells, 2) no staining in cells killed by heat which destroys the enzymes, 3) but staining in cells killed by freezing which does not injure the enzymes.

## *2. Experiments*

Small white navy beans (*Phaseolus vulgaris*, var.?), soaked for twenty-four hours in 1/500,000 or 1/1,000,000 or 1/2,000,000 neutral-red dilutions, germinate at approximately the same rate as do controls soaked in water. The outer coat becomes pink, varying according to the dilution. When the

integument is removed, there is an extremely thin unstained layer; next to this there is the narrow, aleurone layer, brilliantly stained. Further than this there is no staining save on the tip of the radicle.

Seeds are soaked with the coats on, with the coats removed, with the cotyledons separated, and with the cotyledons halved and quartered to determine the part that permeability might have in this differential staining. It is perfectly clear by these experiments that in the cotyledons it is only the aleurone layer that stains, there being a very thin aleurone layer on the inner surfaces of the cotyledons as well as on the outer surfaces. The starch cells of the cotyledon, exposed directly to the stain in small pieces, remain white. Only the protein deposits in the aleurone cells stain with neutral red in these physiological concentrations of the stain.

Beans are soaked in water for twelve hours at room temperature ( $20^{\circ}$  to  $23^{\circ}\text{C}.$ ); excess water is drained from them and they are placed in dry test-tubes; some put into freezing mixtures, some into boiling baths, and some, the controls, simply dried at  $23^{\circ}\text{C}.$  After one hour above boiling point (highest temperature,  $130^{\circ}\text{C}.$ ) and one hour below freezing (below  $-15^{\circ}\text{C}.$  for fifteen minutes of the hour), these two groups of beans and the controls are then soaked in neutral-red solutions of  $1/2,000,000$  for twenty-four hours.

1) The control beans show brilliant aleurone-granule staining and the nucleus is very distinct in each cell as a central unstained region. 2) In the heated beans there is no cellular staining. This is not because of distortion, for the cotyledon cells with their thick walls are not burst nor changed morphologically in any visible way, although the aleurone cells now show brownian movement, which is never seen in any living aleurone cell at this stage (with the light field at least). Macroscopically, there is noted a faint, diffuse pink throughout the heated seeds; this is the ordinary response of dead tissue to a weak dye. There is no concentration nor localization of the dye, so that no color can be detected under the microscope. 3) In the cold-treated beans there is distinct staining of the

aleurone cells only; but the staining is not identical with that in the controls. While most of the stain is concentrated upon the granules, these do not stand out clearly and the nuclear region cannot be distinguished in the general pink. In other words, the very definite localization of the stain characteristic of 'vital staining' is changed, although the concentration of the stain seems normal. Now the stain has undoubtedly spread to the cytoplasm and perhaps to the nucleus.

Two things are known regarding the freezing of plant enzymes, according to Palladin ('23): First, freezing not only does not destroy the enzymes, but it stimulates them. Secondly, "When plants are killed without the destruction of their enzymes the physiological system of the cells appears to become completely disarranged, with the destruction of the interrelations that obtain between the different constituents of the living cell." It is this second phenomenon especially that seems to be visibly demonstrated in the frozen seeds; the diffuse staining in the aleurone cells in which enzyme activity had been initiated and was no doubt somewhat stimulated by freezing shows just this lack of regulation. The whole cytoplasm and possibly the nucleus are now subject to unrestrained hydrolytic action.

*Lupinus alba*, the Italian soup bean, is very favorable material for vital staining. Aleurone-granule staining in this case is a different color from the ordinary neutral red of a neutral to acid range. (See various color manifestations of neutral red in animal cells, Koehring, '30.) The color is darker—a degenerate red approaching a cerise. 1) In early hydration the aleurone granules are small with more space between them than in *Phaseolus* cells, so that staining shows a very definite circumnuclear arrangement of densely colored granules, with the clear unstained nuclear space standing out sharply. This picture is a marked contrast to, 3) the frozen aleurone cells where subsequent staining is intense, but general, so that the granule outlines are blurred. 2) The heated seeds have no cellular staining.

When *Lupinus alba* seeds are stained during the first soaking in 1/1,000,000 neutral red for twelve hours and then subjected to heating and freezing, the controls compare exactly with the frozen for a time. Examined at intervals within one-half hour after freezing, 3) the dead aleurone cells show perfect granule staining with unstained cytoplasm. Shortly after this period, however, staining, although intense, becomes diffuse; after twenty-four hours, there is diminution of color, so that but few cells remain diffusely stained, whereas the controls, 1) now germinating, have distinct, brilliant aleurone granules. 2) The heated seeds never show any stain. In all of these experiments, of course, the controls germinate, the frozen and heated seeds do not.

Most valuable material for these experiments is the little flowering *Lupinus* seed (annual lupine, white, supplied by Hosea Waterer, Philadelphia). In ten or twelve hours the seeds stain and, when placed upon blotting-paper moistened with clear water, they germinate rapidly, becoming tall seedlings in a few days. 1) Throughout germination the aleurone layer remains brilliantly stained and the cells progress through all the stages of vacuole transformation and consequent staining changes which have heretofore been described only by supravital staining methods performed at intervals upon the germinating seeds. When the seed coats burst, the developing chlorophyll of the cotyledons is apparent and at the edges of the cotyledons runs the narrow purplish rim of stained aleurone cells. No diminution of staining activity takes place until the cells have become great, singly vacuolated bodies with no inclusions and little cytoplasm. At all times growth averages that in the unstained controls.

At any time during this germination period smear preparations of the stained aleurone cells may be made and subjected to heat and freezing tests. The temperature extremes used for whole seeds are, needless to say, useless for thin smears of cells. 2) Neutral-red combination in aleurone cells disappears at temperatures far below 100°C.; lack of precise technique very roughly places the reaction between 50° and



80°C. 3) Freezing on the slide until the preparation is a solid mass of crystals reveals very interesting results. In this seed the protein vacuole substance is amorphous and the vacuole contents stain homogeneously, with no sign of brownian movement. Immediately after freezing, the vacuoles present the normal appearance. After an hour or two, however (and this is true for freezing treatment both in smears and in the whole seed), densely red vacuolar precipitates, often in brownian movement, are found in many cells. Such cells do not show stained cytoplasm for considerable periods, in contrast to the more usual type of cell found among them, where the whole cell is diffusely stained.

In the pea, *Pisum sativum*, the aleurone granules are not in cells devoted exclusively to these protein deposits, but in cells also containing some starch granules. 1) Neutral-red staining in these cells is normally confined to the aleurone granules only. 3) In the frozen cells all of the granules seem to be stained as well as the cytoplasm in general. Such a condition may demonstrate this: In the bean and lupine no proteolytic action in starch cells is initiated in early hydration; there is consequently never any neutral-red staining in starch cells. In the pea cells, however, staining, which is normally nicely regulated to the protein constituent, spreads, in the cell killed by freezing, to other cellular elements, controlled, undoubtedly, by an altogether different enzyme system. Palladin ('23) recognizes that in dead cells there is no regulation of one enzyme by other enzymes, and that proteolytic enzymes may decompose other enzymes (Petrushevskaja, '06-'07).

### *Summary*

Plant enzymes are not injured by freezing temperatures; they are destroyed by high temperatures. Neutral-red staining, occurring in aleurone cell vacuoles containing protein deposits in the process of hydrolysis, can be concentrated by and remains in frozen cells; it cannot be concentrated by and is destroyed in heated cells. Again, enzyme reactions and neutral-red staining reactions closely parallel each other in response to temperature.

ARTIFICIAL PARTHENOGENESIS IN STARFISH EGGS INDUCED BY  
MOMENTARY RAISING OF THE TEMPERATURE TO  
AN ENZYME OPTIMUM

Starfish eggs develop parthenogenetically when subjected for brief intervals to temperatures between 33° and 40°C. with a rather definite optimum exposure at 35°C. for seventy seconds (Lillie, '08). Below these temperatures there seems to be insufficient stimulation and no membrane formation nor subsequent development takes place. Up to 45°C. membrane elevation is stimulated, but protoplasmic injury prevents development. The activation of other marine eggs by these temperatures has also been described (Just, '15, and others)

Enzyme action, as the mechanism for this response to momentary temperature elevation, is repeatedly suggested by Lillie as in the following quotation: "... the assumption of some process accelerated by an enzyme with an optimum temperature of 38° to 40° and rapidly destroyed at 45° would account for the above relations."

Repetition of these experiments, following the directions given by Lillie, proves this a dependable method of artificial parthenogenesis, usually producing a high percentage of swimming larvae. The relationship of this reaction, so strongly suggestive in itself to enzyme activity at its optimum, to accompanying neutral-red phenomena is as follows:

Ripe starfish eggs cannot be visibly affected *in vivo* with neutral red in any physiological solution, that is, dilutions in which the adult stars are brilliantly stained for long periods with no harmful results. 1/500,000 and 1/1,000,000 neutral-red solutions stain adults so that after one hour in these dilutions the tube feet, dermal branchii, and pedicellaria are bright red. The walls or supporting tissue of the ovary may become stained, but the eggs shed from these plumes show no immediate trace of the stain.

After stars are stained for a period of several hours, they are washed in clear running sea-water for some hours or days, rinsed in fresh water (as are all dishes and instruments as a precaution against the presence of sperm), and the eggs are

allowed to shed into sea-water from the dissected plumes. The eggs are then transferred to fresh sea-water. It is certain that there is no trace of stain in the medium. Upon heating these colorless eggs according to Lillie's directions and watching membrane elevation and subsequent development, a neutral-red phenomenon is noted.

With the first appearance of the fertilization membrane as a loosely adherent, crinkly structure, a deep pink color is noted between the cortex and the lifting membrane. This clear, intense pink remains as the membrane stretches, smooths, and is further lifted from the egg. Imperceptibly the cortex also becomes stained as a delicate red line. No granules or other egg contents are stained. The fertilization membrane itself is not red.

Within an hour, as maturation is completed, the pink color usually disappears. If the egg is eccentric within the membrane, the widest area becomes colorless first. Dilution and diffusion as water is absorbed may be the factor in the disappearance of the color. The cortex loses color slowly; blastomeres often show the delicate line of red.

Unheated control eggs from stained stars have not been observed to develop any color. A wavy-looking thin membrane sometimes noted among starfish eggs—never to be confused with the smooth fertilization membrane—is not stained. No pink halo or other color effect beyond the cortex is noted.

A ray effect from cortex to fertilization membrane is very striking in the heated eggs. Whether the ray effect is stimulated more than normally or merely rendered more visible by the neutral red is not known for these eggs. All stages from very delicate pink ray effects to much coarser amoeboid processes from the egg are noted. These must be regarded as secretion products from the egg. The lifting of the fertilization membrane appears to be due to an extrusion or secretion from the egg. That this egg secretion is colloidal would be expected. That the secretion is of such a nature as to render infinitesimal quantities of neutral red visible must be admitted.

When membranes are formed at temperatures above the optimum favorable for subsequent development, little increase in color can be detected. When the eggs are killed by a temperature of  $43^{\circ}$  for seventy seconds, no color is seen, although some membranes are elevated. When by short exposure at such temperature,  $43^{\circ}$  for fifteen seconds, the eggs are not killed and the percentage membrane elevation is high, the pink color brought out is not greatly increased above the color noted at the optimum conditions for development.

Experiment 7 is reported in the notes as typical to give details of these reactions. No explanation for the presence of neutral red in the carefully washed eggs is available other than that the stain, in small quantities, entered the egg during the staining period. A ripe egg is very inert; no hydrolytic processes occur; therefore no neutral-red reaction would occur. Upon shedding the eggs into sea-water, however, slight cortical secretion is stimulated. This is so slight as to be insufficient to demonstrate the presence of neutral red. Greater stimulation of secretion induced by an agent of parthenogenesis—heat in this instance—may produce such a quantity and activity of an enzyme that every available molecule of neutral red is combined and thereby made visible. It is significant that Marston's ('23) neutral red-enzyme precipitation experiments, to produce maximum effect, were carried on at  $40^{\circ}\text{C}$ .

Starfish eggs stained supravitaly—that is, the adult stars are not stained, but the eggs are shed into neutral-red solutions—color readily. In all dilutions of the stain unheated eggs are obviously less brilliantly stained than the heated eggs in the same dilutions. Degrees of change in the cortex and secretions from the cortex are revealed in striking manner by using a series of temperature exposures and also series of varying stain dilutions. Stimulation and control of the cortex activities to what must be approximately normal secretion, if the resulting swimming larvae are to be taken as an index of comparatively normal development, as well as hypersecretion at the expense of the egg are indicated in these experiments.

As a 1/500,000 solution of neutral red, for instance, as well as the other dilutions used in the experiments, each produces, at the optimum of 35° for seventy seconds, perfect fertilization membranes and a very definite type of secretion and cleavage specific for each dye concentration, these experiments will not be further discussed here, for the observations bring out fundamental inhibition-stimulation effects of the dye itself which are not relevant for purely temperature phenomena indicated by the dye. They will be discussed in a later paper concerning neutral red as an agent of artificial parthenogenesis.

Very tentatively, Koehring ('30) suggested that neutral red would indicate the presence of proteolytic enzymes upon the surface of ripe eggs when the egg secretion was protected by a jelly layer; also, that the sperm carries a stronger proteolytic enzyme; also, that neutral red acts as an enzyme stimulant. If the above conditions are valid and if the egg is activated mainly by an enzyme effect, as from the sperm, then neutral red might be an agent of artificial parthenogenesis in its enzyme-stimulating capacity. Such is found to be the case in starfish eggs. Neutral red in a definite concentration range effects membrane elevation and varying degrees of cleavage in unfertilized eggs at normal temperatures, in the light, but not in the dark. As the effect of activation by neutral red is not a temperature reaction, details of the experiments have no place in this paper, though mention may here be made of the fact as contributing evidence to the early suggestions of an enzyme phenomenon involved in artificial parthenogenesis by heat.

While it is as yet by no means proved that ripe eggs shed a purely proteolytic substance which, at its optimum, is an activating agent, there is strong evidence that there is a hydrolytic enzyme involved. Lillie says ('08, p. 385), "The above temperature relations appear to indicate a dependence on some enzyme action." He suggests, however, not a proteolytic, but a lipolytic enzyme: "Certain hydrolytic cleavages may be concerned, possibly a saponification resulting in

a partial solution of the surface layer." That the enzyme involved is primarily lipolytic does not agree with most in-vivo neutral-red phenomena nor with the biochemical reactions of the dye. However, Marston has found that traces of lipolytic enzymes existing in connection with proteolytic enzymes are also precipitated by neutral red.

### *Summary*

The neutral-red phenomena, in connection with temperature reactions in fertilization-membrane elevation, support suggestions that ripe starfish secrete a weak proteolytic enzyme and that stimulation of the enzyme effect to its optimum will activate the egg. In other words, if the ripe cortex of the unfertilized egg, which secretes only enough substance to slightly injure adjacent sperm, be stimulated to secrete the full quota of extrusion called out normally by secretion of the sperm, or, experimentally, by a large number of chemical and physical agents including temperature elevation, then consequent dehydration of the cortical region is effected and the train of events producing cleavage has started. The facts are: this secretion is responsive to the neutral-red reaction, and is a thermolabile substance acting in the same range as do the majority of proteolytic enzymes—which are points of evidence as to its enzyme nature. (Further evidence that this substance responds to  $\text{CO}_2$ , KCN, lipoid solvents, saline or hypertonic effects, etc., in precisely the same manner as do other substances of cytoplasmic origin universally staining with neutral red and known to be proteolytic, is forthcoming as additional evidence of the enzyme nature of egg secretion.)

### NOTES

#### *Experiment 7. Asterias eggs*

8/22, A.M. Adults stained in 1/500,000 neutral red for twenty-three hours (six stars in 10 liters water). Temperature,  $22^\circ\text{C}$ .

8/23, A.M. Adults removed from dye and washed in running seawater for two and one-half hours. Very brilliant and active. Tube feet stick tight to the glass and many must be torn in removing stars

from aquarium. (Animals not dissected kept for one week, remaining in good condition as color gradually fades.) Rinsed and dissected at 11.30 A.M. Shed eggs washed at 11.35. Temperature, 21°C.

Lot I. 11.35. Not treated.

Lot II. 11.45. 32° for seventy seconds.

Lot III. 11.47. 35° for seventy seconds.

Lot IV. 11.53. 38° for seventy seconds.

Lot V. 11.58. 43° for seventy seconds.

Lot VI. 12.02. 35° for seventy seconds.

Lot V. 12.09. A few fertilization membranes. No color. Great swelling. All eggs dead.

Lot VI. 12.12. High per cent fertilization membranes, still somewhat crinkly. Space between cortex and fertilization membrane pink. No color in eggs. Pink beneath membranes much deeper than eggs in 1/1,000,000-treated adults. Maturation proceeding.

Lot IV. 12.27. Many membranes; still crinkly. Pink space between fertilization membranes and cortex. Some distortion of eggs. Maturation proceeding.

Lot III. 12.34. High per cent perfect fertilization membranes, smooth and spherical. Eggs spherical. Pink area between membrane and cortex. Pink cortex. Maturation.

Lot II. 12.45. Only one fertilization membrane noted in entire lot; has pink area between membrane and cortex. A faint pink edge to the egg membrane is not certain in the majority of eggs. Eggs perfectly spherical. Maturation.

Lot I. 12.47. No fertilization membranes. No color. Maturation.

Lot II. 1.33. No fertilization membranes noted. Both polar bodies detached. Barely perceptible pink border on egg.

Lot III. 1.36. Perfect fertilization membranes and eggs. Borders of all eggs decidedly pink, rather deep into the cortex in some eggs. This is a bright, delicate pink; no granules are stained—only a fluid (?) stained. Very delicate ray effect in pink area between cortex and fertilization membrane.

Lot IV. 1.44. Many have not matured. Fertilization membranes still crinkly in some cases and not very far lifted from the eggs. Many eggs aspherical and otherwise distorted. Pink area persists between cortex and membrane. Pink cortex.

Lot VI. 1.55. Like II.

Lot I. 5.15. No cleavage. No color.

Lot II. 5.17. No cleavage. No color.

Lot III. 5.19. High per cent cleavage. Most of it quite regular. Mostly in morula stages, but there are also thirty-two-, sixteen-, eight-, and a few four- and two-cell stages. No staining in blastomeres, but pink edge is so deep as to give effect of black cortex. Polar bodies seem very prominent. No color under fertilization membrane.

Lot IV. 5.27. No cleavage. Pink cortex brilliant. All transitions between ray effect and secretion of coarse granular material into area under fertilization membrane. Numerous little clumps of these granules.

Lot VI. 5.32. High per cent cleavage, mostly morula stage. Blastomeres have pink membranes. No pink in area outside embryo.

Lot VI. 7.57. High per cent blastulae. No color. Some abnormalities.

Lot III. 8.02. Many perfect blastulae. Some abnormalities. No color.

8/24. Lot III. Swimming larvae. No color.

Lot VI. Swimming larvae. No color.

#### TEMPERATURE RELATIONSHIPS IN THE STAINING OF PALAEMONETES AND COMPARISON WITH TEST-TUBE REACTIONS OF NEUTRAL RED PLUS PEPSIN

A macroscopic study of neutral-red staining in the transparent marine prawn, *Palaemonetes*, reveals facts concerning the fate of neutral-red dye in temperature reactions. What is the explanation for the 'disappearance' of the dye at critical temperatures?

*Palaemonetes* adults and the later stages preceding the last molt stain in 1/250,000 to 1/1,000,000 dilutions of neutral red with no appreciable harmful effects. Feeding, molting, and all movement in the stained animals compare favorably with these activities in the controls for many weeks. With the greater staining dilutions the only color visible macroscopically is in the contents of the two stomach divisions, cardiac and pyloric. In 1/500,000 and 1/250,000 concentrations cellular staining in the digestive glands, gills, and carapace may be macroscopically detected and distinguished from any natural pigmentation, of which a little is always present even in the most transparent male individuals.

The stomach in these Crustacea comprises two distinct, large, muscular chambers, and the quantity of dye which both concentrate from dilute solutions produces a striking intensity of red color. The animals are stained for twelve to twenty-four hours in the dye solutions, rinsed several times, and kept in fresh sea-water which is changed every day and also after



feeding. The brilliant color in the stomach is not lessened for many days, although no trace of the dye is present in the medium after the first twenty-four hours. For example, animals stained August 11th in 1/250,000 dilution for twenty-four hours have brilliant and very large stomachs on August 18th. This apparent increase in size of the stomach is probably due to the intense staining of the digestive-gland tissue surrounding the stomach which cannot be separately distinguished macroscopically when both glands and stomach lumina are densely stained.

The color in the lumen of neither stomach chamber can be appreciably diminished by feeding over long periods. For example, animals stained August 7th for twenty-four hours in 1/500,000 neutral red and thereafter kept in fresh seawater ate voraciously of fish scraps on August 11th for one hour. As the white, opaque fish flesh was stuffed into the cardiac stomach this organ became almost white for a short time. But very soon, the ingested food became more and more pink, until by the end of the feeding period both the cardiac and pyloric stomachs were as brilliant as before feeding. On August 16th these same animals' stomachs were still a clear, unmistakable pink neutral-red color, and noticeable fading of the color began on this day, so that the stomachs became the original transparent to pale brownish color eventually.

When the stained stomachs are empty of food, as when the animals are not fed for several days, they are a very brilliant transparent red or pink. When such animals are stimulated by the odor of food in the water, but are not allowed to reach the food, the stomachs churn noticeably, as during the digestion of food. No heightening of the color of the lumen contents can be observed in this period, presumably of enzyme stimulation. Also after the digestion of a large meal as the red stomachs become less opaque, the color, while it becomes transparent, is not noticeably diminished in intensity, although this must be a period of decreased enzyme activity. These results are, of course, disappointing and lead the author to think that, while the animals seem to suffer in

no way, there must be an abnormal hyperenzyme state in the stomach stimulated by neutral red. Perhaps the presence of neutral red is a constant stimulus for enzyme secretion. The dye molecules in the lumen fluids must be utilized over and over again in combination with and release from the enzyme. Also, dye molecules entering the body fluids and tissues during the staining period must be recalled into the digestive tract. There is evidence for this in *Daphnia* reactions as described by Koehring ('30) and also in the medical use of neutral red as a test for hyper- and subacidity and enzyme content in the human stomach (Winkelstein and Marcus, '29).

Now to proceed to the temperature reactions of these quantities of neutral red in stomachs which may be considered as very small test-tubes of enzyme plus neutral red: When *Palaemonetes* are subjected to one-minute exposures to temperatures above 40°C., there is visible tissue change: the transparent, colorless flesh becomes opaque. Against this opaqueness, the tissue staining, best seen in the gills, and the stomach staining remain macroscopically distinct up to about 50°. Above 50° the opaqueness of the tissues totally obscures staining, but when the carapace is removed it is plain, under a dissection binocular, that cellular neutral-red bodies in the gills are not changed much before 64°. Between 64° and 68° distinct neutral-red bodies disappear. But color does not totally and wholly disappear—the gills become a diffuse, dull faded red. Above 70° this diffuse color rapidly fades, so that at higher temperatures the gills become white very quickly.

The stomach likewise becomes invisible at temperatures higher than 50°, due to the opaqueness of the tissues, but when the killed animals are torn open it is noted that below 60° the stomach contents are a bright, clear red coagulum. Somewhere above 60° the shade of the color changes to a dull-looking red. There seems to be a change not so much in the amount of the color present, but in the condition of the color or the state in which it is held. At 70°, 80°, 90°, 100° this dull, almost old rose persists in the stomach contents. Above 100° the color can only literally be 'boiled out' at exposures of from five to ten minutes.

Now some test-tube reactions of neutral red and enzymes are as follows: First, boiling neutral-red solutions in no way changes the dye or injures it. Secondly, boiling a mixture of neutral red and pepsin in no way changes the color of the original solution nor injures the dye. That is, heat has no effect on the dye, nor has heat plus enzymes.

Thirdly: Pepsin in a 1/80,000 solution of neutral red (or any other dilution which is light enough in color to make this reaction striking) is allowed to stand overnight. A fine precipitate is formed, so densely red as to appear black. (Old pepsin which has become inactive will not form a precipitate.) A drop of the solution containing some of the finest particles of the precipitate, studied under the microscope, is very illuminating as to the nature of vital staining. The thin film of weak dye appears quite colorless: the fine particles of precipitate are of the color of intensely staining cellular neutral-red bodies. There is no mystery in this part of the mechanism of azine vital staining—the dye is concentrated by the enzyme colloids. It is difficult to imagine strong proteolytic enzymes free and diffuse in the cytoplasm in living cells; hence the invariable localization of vital-staining substances in living cells. But no matter how small the cellular vacuole or granule containing or producing the enzyme, if the dye can reach it, the brilliancy of the precipitate formed in or upon it is an index of the quantity of an active enzyme.

The neutral red-enzyme precipitate is but slightly soluble in water. It may be washed free from the excess dye and the enzyme impurities which are probably in the quantities of a sticky black substance adhering to the filter-paper. The washed precipitate does not color the water in the test-tube even after standing many days. But when a test-tube of clear water containing neutral red-pepsin precipitate is heated over 60°, the water gradually becomes pink and the precipitate disappears. The dye is released from combination with the enzyme when the enzyme is inactivated. (Marston gives other examples of the release of the dye from the enzyme.) The dye is in no way changed; it is only freed and it diffuses in the medium.

This is what happens in cellular vacuoles. In heat death in the cell there may be changes in permeability, water-lipoid phases, etc., but as long as the enzyme is uninjured there can be no release of the enzyme-dye combination as it exists in a 'neutral-red body.' But at a critical temperature, far above that of heat death of the cell, the enzyme is injured and the dye is released. The free dye diffuses so rapidly in the dead cell that it becomes invisible almost immediately. It is an infinitely small amount of dye that is present in the most brilliant concentrations of cellular organs in comparison with the size of the cell, and, in dead cells particularly, diffusion of the freed dye would be extremely rapid at high temperatures.

This certainly accounts for the startling disappearance of neutral-red staining in cells at high temperatures. But the stomach of the prawn is a different matter. Quantities of dye are obviously stored in the large stomach. Release of the dye from enzyme combination at a critical temperature would not in itself remove the dye. It is there until it can diffuse out. The color change, from the brilliant red of the precipitate concentration to a dull color, indicates release of the dye from combination and also diffusion as far as the limits of the stomach walls allow. Rapid diffusion is impossible, however; even when the coagulum of stomach contents is dissected out into water, the color disappears slowly. It takes longer for a cube of crystallized sugar to dissolve than for one crystal to dissolve.

It was noted that in the gills of *Palaemonetes*, observed in bulk, all color did not disappear immediately with the distinct neutral-red bodies, although rapid diffusion is possible in this tissue. But the stomachs of the animal are like little test-tubes holding considerable quantities of dye-enzyme precipitate; release of the dye from combination is indicated, just as in a large test-tube, but the color does not disappear.

### *Summary*

The reaction of neutral-red staining in *Palaemonetes*, considering particularly the stomach contents, in response to temperatures critical for enzyme inactivation, parallels the reaction in the test-tube of the neutral red-pepsin precipitate in response to these temperatures. It is extremely probable that all vital staining phenomena may be duplicated in the test-tube when the relationship of the dye to enzymes is more thoroughly understood.

### CONCLUSION

Neutral-red staining in both animal and plant material closely approximates in its reactions to temperature the reactions of enzymes to temperature. Falk ('22) has said:

The chemical study of enzyme actions as ordinarily carried on consists almost entirely of the investigation of the actions of enzyme preparations on substances in vitro. While enzyme studies can be carried on to a certain extent in vivo, the amount of this kind of work has been comparatively small, and although of the greatest interest, has been more in the nature of testing hypotheses and conclusions obtained from the work in vitro where more accurate control of the conditions was possible.

Neutral red must be thoroughly tested as an enzyme indicator, for the possibilities of its use as a tool for the investigation of enzyme activity in vivo seem very promising.

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# THE ANATOMY OF DIPLODINIUM MEDIUM

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SEVEN TEXT FIGURES AND TWO PLATES (THIRTEEN FIGURES)

## AUTHOR'S ABSTRACT

In this paper evidence is given in support of the author's statements (Rees, '30) that in ciliates in the genus *Diplodinium* a neuromotor apparatus, as described and figured by Sharp ('14), could not be differentiated. For the study of minute anatomy ciliates were cut into serial sections  $3\mu$  thick. The body which in *D. medium* appeared to correspond to the motorium of *D. ecaudatum* was shown to be a fold of the middle layer of ectoplasm. Fibers, described and figured by Sharp as connecting the motorium to the organelles of locomotion and of food taking and to a ring about the esophagus, could not be differentiated. Other fibers which Sharp designated as opercular were shown to be part of a fibrillar complex extending throughout the middle layer and the inner boundary layer of ectoplasm. The walls of the esophagus were shown to be formed by the fused middle and inner layers of the ectoplasm. The esophageal fibers therefore belong also to the above-mentioned fibrillar complex. In *D. medium* a system of esophageal membranelles was differentiated, described, and figured. The ciliary systems of *Paramecium ecaudatum* and of *Diplodinium medium* were shown to be strikingly similar. The principle of descent from a common ancestor adequately explains the above similarity, but is out of harmony with the discrepancies between Sharp's account of *D. ecaudatum* and this account of *D. medium*.

• In 1914, Sharp reported the occurrence of a neuromotor apparatus in *Diplodinium ecaudatum*, a ciliate occurring in the rumen and the reticulum of cattle. This apparatus was said to resemble the nervous systems of some of the lower Metazoa, and it was suggested that it functions in this unicellular organism in a manner similar to that of the nervous system in multicellular organisms, i.e., in the transmission of stimuli to various organelles of locomotion and of food taking, and thus in the coordination of movements of these organelles.

If confirmed, this work must be accepted as a demonstration of the development of a nervous mechanism without cell multiplication (cleavage) or the formation of germ layers. Sharp's paper has been cited in practically all works of reference in protozoölogy and his figures have been copied in many of the text-books on this subject and in several text-



books of general zoölogy. However, sufficient work has not been done by other investigators to lead either to confirmation or to refutation of his thesis. Dogiel ('27) concurred in Sharp's position without publishing the data. The present writer ('30) questioned Sharp's interpretation, but failed to publish figures or to give sufficient details. A number of letters from various laboratories in the United States and in foreign countries have been received at this laboratory requesting a more detailed report.

In the preparation of this report it was soon realized that there is a long list of species which have been proposed in the genus *Diplodinium*, new specific names having been added by every investigator who has conducted a taxonomic study. Since in this locality no taxonomic work on the genus has been done, it was difficult to determine positively whether the present writer was dealing with the same species as those studied by Sharp ('14). Therefore *Diplodinium medium* was selected rather than *D. ecaudatum*, because in the former species the relative positions of the adoral and the dorsal membranelles, and the shape of the macronucleus, make it possible to classify individuals with certainty. Furthermore, there occurs in *D. medium*, in the well-defined middle layer of the ectoplasm (*M.E.L.*, text figs. 1, 2, 3, 5, and 6, and pl. 2, figs. 7 to 13), a fold forming a body resembling in size, shape, and position the motorium of *D. ecaudatum* Sharp 1914.

#### REVIEW OF THE LITERATURE

As described and figured by Sharp ('14), a deeply staining mass, the motorium, situated within the ectoplasm at the anterior end of the body of *D. ecaudatum*, was thought to correspond to the metazoan brain. Fibers were described and figured running out from the motorium to the ciliary rootlets of the cirri, which are the organelles of locomotion and of food taking. One fiber was said to extend from the motorium to the esophagus, around which it formed a ring resembling the nerve ring of some of the Metazoa. Sharp maintained that the position of this mass and the above-mentioned fibers

within the body and the relationships thereof to the other organelles of the body did not suggest functions either of contraction or of support. Functions of conduction and of coordination, therefore, were assumed. Lynch ('29, '30) described neuromotor systems in four newly discovered species of ciliates from the digestive system of *Strongylocentrotus*, a sea urchin. He cited six previous authors who have described neuromotor systems in ciliates. The inclusion of four additional papers (Yocom, '18; Taylor, '20; Rees, '22, and Dogiel, '27) would have practically completed the list of references on this subject.

The position taken by the present writer ('30) was as follows: 1) That the structure which Sharp described as the motorium is a fold of a layer of the ectoplasm; 2) that this layer is also folded beneath the pellicle in some of the lips and furrows which occur at the anterior end of the body; 3) that Sharp misinterpreted sections through this layer as strands; and, 4) that the above layer forms a cylinder which Sharp interpreted as a ring around the esophagus. Hence the conclusion was reached that the conditions set forth by Sharp for a coordinating nervous mechanism in *Diplodinium ecaudatum* do not appear to be fulfilled. As stated above, the object of the present paper is to supply figures and other data to substantiate this conclusion.

#### TECHNIQUE

While working on the morphology of *Buxtonella sulcata* the writer ('30) developed and described a method for orienting and sectioning individual ciliates. For this purpose they were embedded in small blocks of paraffin which was stained with sudan III, the red blocks being in turn embedded in unstained paraffin of lower melting-point. This technique was also used in the studies for the present paper on *Diplodinium*, but has been improved as follows:

In the previous work the melted, unstained paraffin, which was contained in a Syracuse watch-glass, was allowed to cool to form a hardened surface film before the red blocks were

embedded in it; however, such cooling is unnecessary, since the red blocks may be embedded precisely as though they were organisms; the occurrence of air bubbles may thus be avoided. The method of Zirkle ('30) of dehydrating plant tissues in ethyl alcohol, and of running them thence into 100 per cent N-butyl alcohol from which they were embedded into paraffin, has been used with success by the writer for ciliates. It is superior to the method previously used, involving ethyl alcohol, xylene, and cedar oil.

About eighty individuals have been sectioned and these include the following preparations: *Diplodinium ecaudatum* Fiorentini 1889, three series containing twenty-five to thirty longitudinal sections at  $5\mu$ , two series at  $3\mu$ , and several incomplete series at  $3\mu$ ; *Diplodinium medium* Awerinzew and Mutafova 1914, four series containing twenty-five to fifty-two cross-sections, one series of forty-three oblique sections, and six series of twenty-five to forty longitudinal sections, all at  $3\mu$ ; and *Diplodinium maggii* Fiorentini 1889, six series of twenty-four to forty longitudinal sections. Serial sections of 8, 10, and  $12\mu$  through individuals of these several species have also been prepared. The ectoplasmic layers could best be followed and their relationship determined in thin sections. As indicated by the title of this paper, the drawings are of *D. medium*, but the photomicrographs include all three species.

#### THE GENUS DIPLODINIUM

Dogiel ('27) has published an excellent monograph on the genus *Diplodinium* which he divided into four subgenera. He contends that the genus *Metadinium*, which was proposed by Awerinzew and Mutafova ('14), should be sunk in favor of the genus *Diplodinium*; the present writer is of the same opinion. It appears also that the genus *Epidinium* Crawley 1924 should likewise be discarded for the genus *Diplodinium*. This genus should thus include all ciliates which possess two spiral rows of cirri at the anterior end of the body, which is otherwise devoid of cilia. One of these rows surrounds the

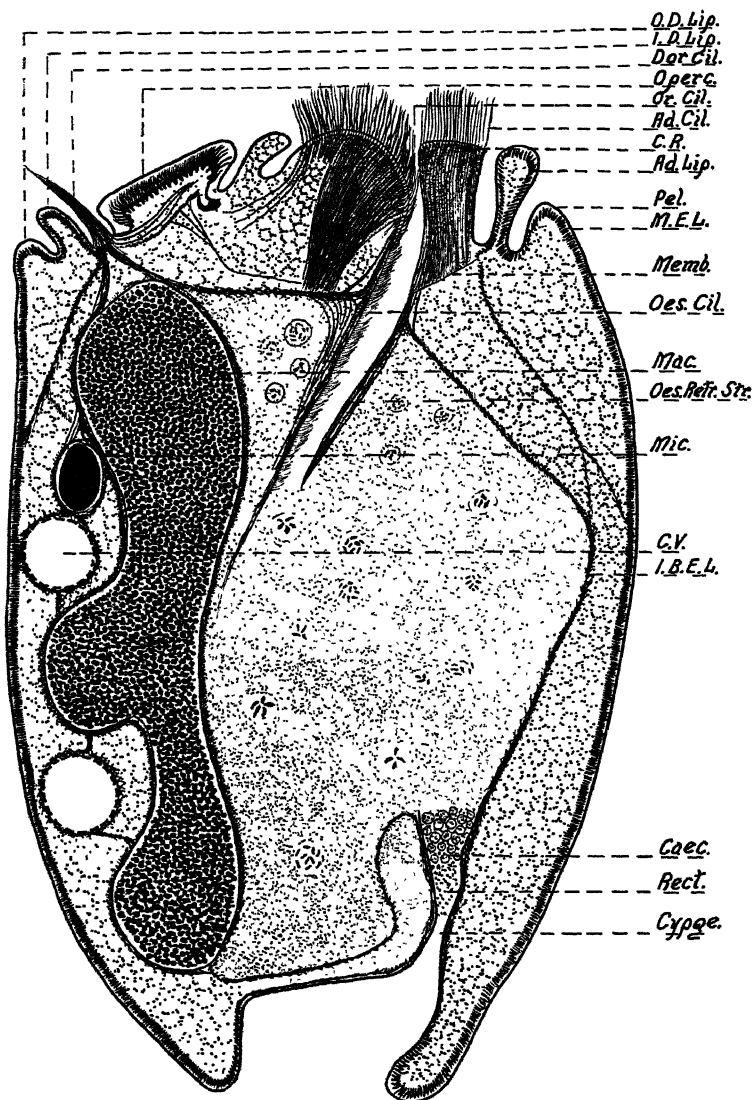
mouth and the other surrounds a pit-like depression. As was pointed out by Dogiel ('27), the number and position of the skeletal laminae and the shape and position of the macronucleus furnish a basis for the subdivision of the genus into species. The three species mentioned in the preceding paragraphs have been thus determined.

#### GENERAL ANATOMY OF SPECIES OF DIPLODINIUM

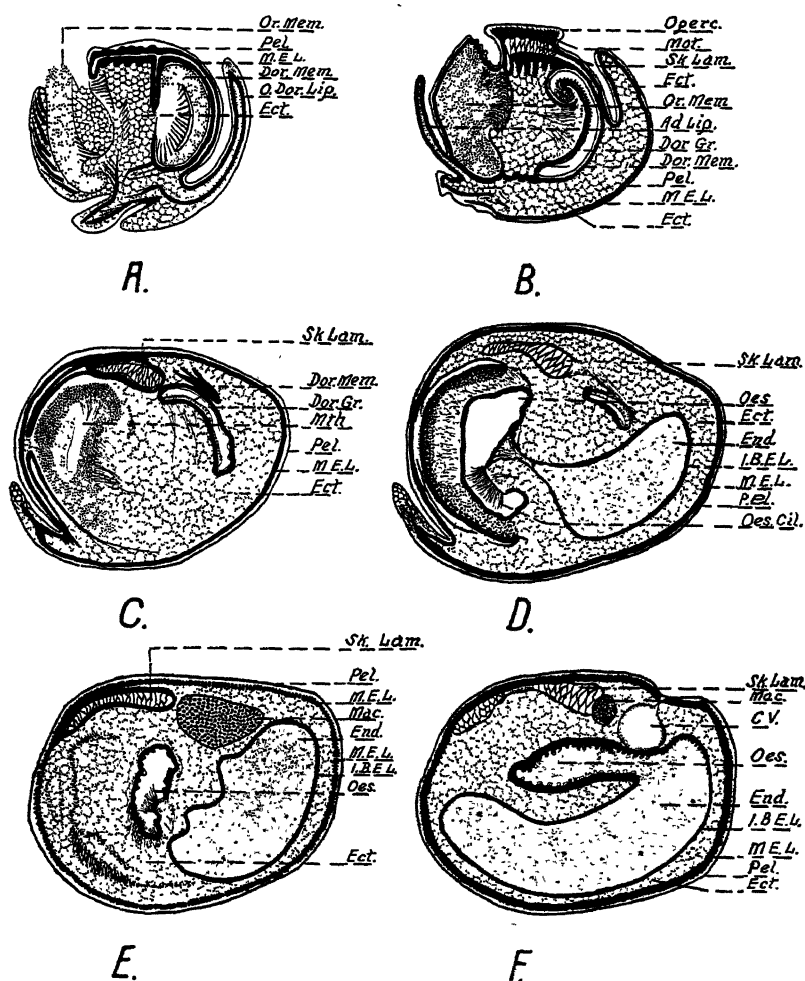
Sharp's ('14) excellent account of the anatomy of *Diplo-dinium ecaudatum* needs very little revision. In fact, with slight modifications his description of this species is equally well suited for the other species of the genus. It will be necessary, therefore, only to point out these modifications and to indicate the points of disagreement between his interpretation and that of the present writer.

Sharp's ('14) figure 3, plate 4, of *D. ecaudatum* should be compared with the present writer's text figure 1 of *D. medium*. Both of these drawings are semidiagrammatic and show most of the important structures and organelles. The writer has followed Sharp ('14), who represented the ectoplasm by fine alveoli and thus differentiated it from the endoplasm. However, careful microscopic study has failed to reveal any marked differences between the endoplasm and the ectoplasm except that food bodies do not occur in the latter. As was pointed out by Awerinzew and Mutafova ('14) and by Dogiel ('27), the macronucleus of *D. medium* resembles a reversed letter E (text fig. 1); in *D. ecaudatum* the above organelle is elongated and resembles a banana. In *D. medium* there is only one skeletal area, which divides caudally into two forks (text fig. 2, *Sk.Lam.*); in *D. ecaudatum* there are three such areas.

Other noteworthy differences between the two species are as follows: In *D. medium* there is a very prominent layer of the ectoplasm which the writer has called the middle layer (*M.E.L.*, text figs. 1, 2, and 3; pl. 2, figs. 7 to 13) and which, except in the region of the adoral lips, lies directly beneath the pellicle. It will be noted that this layer is composed of



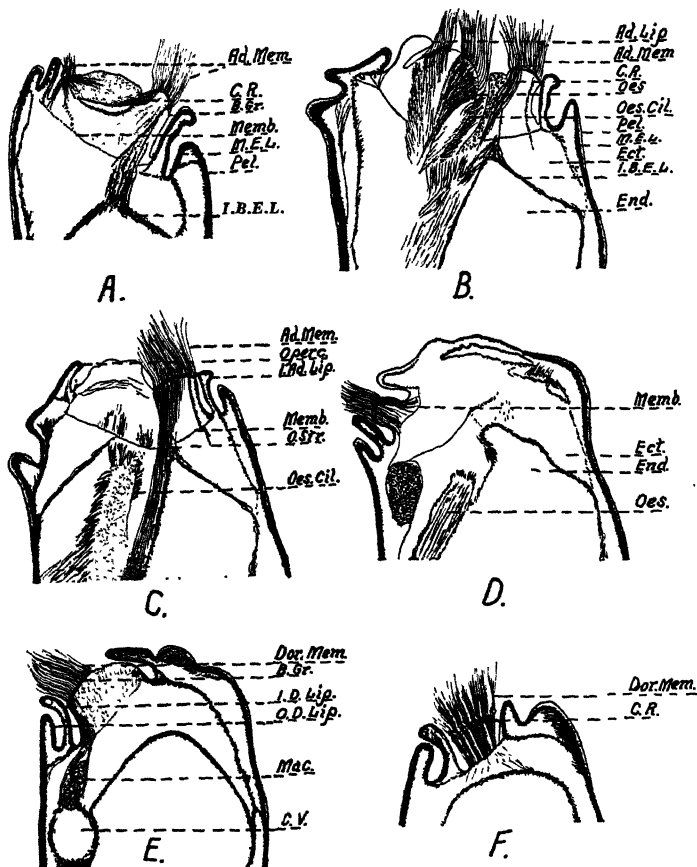
Text fig.1 Semidiagrammatic longitudinal section through *Diplodinium medium*.  $\times 450$ . The macronucleus, the micronucleus, and the contractile vacuoles occur within the ectoplasm; they may, therefore, not be found in a thin section through the center of the body, such as the section figured. *O.D.Lip.*, outer dorsal lip; *I.D.Lip.*, inner dorsal lip; *Or.Cil.*, oral cilia; *Ad.Cil.*, adoral cilia; *Ad.Lip.*, adoral lip; *Operc.*, operculum; *Dor.Cil.*, dorsal cilia; *C.R.*, ciliary rootlets; *Memb.*, membrane; *Pel.*, pellicle; *M.E.L.*, middle layer of the ectoplasm; *Oes.Cil.*, esophageal cilia; *Mac.*, macronucleus; *Mic.*, micronucleus; *C.V.*, contractile vacuole; *Oes.Refr.Str.*, esophageal retractor strands; *Caec.*, cecum; *Rect.*, rectum; *I.B.E.L.*, inner boundary layer of the ectoplasm; *Cypge.*, cypotype.



Text fig. 2 Camera-lucida drawings of six cross-sections of *Diplodinium medium* from a series containing fifty-two sections of  $3\mu$  stained in iron haematoxylin. Section D,  $\times 525$ ; other sections,  $\times 450$ . Section A, of serial section no. 4, through the anterior end of the oral and the dorsal membranelle zones. Section B, of serial section no. 6, just cephalad of the mouth or cytostome. Section C, of serial section no. 8, near the mouth. Section D, of serial section no. 10; note the enlargement of the esophagus and the connection between the middle and the inner layers of the ectoplasm. Section E, of serial section no. 12, through the anterior end of the macronucleus. Section F, through the anterior contractile vacuole; note that the walls of the esophagus are continuous with the inner layer of the ectoplasm. *Sk.Lam.*, skeletal laminae. Other abbreviations as in text figure 1.

an interwoven network of fine fibers (see especially text fig. 7). In *D. ecaudatum* this middle layer is not so prominent and hence not so easily followed. Sharp described it as a layer of fine alveoli.

The disagreement between the writer's interpretation and the interpretation of Sharp ('14) has to do primarily with



Text fig. 3 Diagrams of the ectoplasmic layers, the cilia, and the ciliary rootlets of six longitudinal sections of  $3\mu$  from a series of forty-one serial sections through *Diplodinium medium*.  $\times 525$ . Section A, of serial section no. 9; section B, of serial section no. 13; section C, of serial section no. 17; section D, of serial section no. 22; section E, of serial section no. 24; and section F, of serial section no. 27. The adoral and the oral membranelle zones are shown in sections A, B, and C; the dorsal membranelles, in sections D, E, and F. B.Gr., basal granules. Other abbreviations as in text figure 1.

this layer and with the inner boundary layer of the ectoplasm. The pellicle and the middle and inner layers of the ectoplasm are described in detail in the following paragraphs.

#### THE PELLICLE

Reference to text figures 1 and 6 shows that the pellicle covers the entire surface of the body and forms the lining of the mouth and the esophagus as well as of the anus, the rectum, and the cecum. It may thus be compared to the epithelium of metazoan organisms, but differs from the latter in lacking continuity, being interrupted at the distal end of the esophagus to permit the entrance of food into the endoplasm and also in the cecum to permit the exit of waste material. As in other ciliates, the pellicle appears to be a thin, transparent, flexible membrane, resembling in this respect the cuticle of the invertebrates. Reference to text figure 2, sections D, E, and F, shows that in the esophagus of *D. medium* the pellicle is folded. The present writer ('30) described these folds as 'longitudinally extending, accordion-like pleats.' This folding suggests that marked distention of the esophagus is possible, the pellicle being, perhaps, relatively inelastic.

#### THE MIDDLE LAYER

The middle layer of the ectoplasm lies directly beneath the pellicle except where the ectoplasm is greatly thickened at the anterior end of the body. In a zone beneath the adoral groove this layer is reflected back upon itself and appears somewhat as would a section through a partly inverted glove finger. It will be noted that the adoral region is situated ventrally at the extreme anterior end of the body. On the dorsal side of the adoral region the reflected middle layer must, therefore, pass beneath the operculum, but the pit-like depression beneath the dorsal membranelle zone pierces this layer, forming a circular opening. A short distance caudad of the dorsal membranelle zone, part of the layer appears to fuse with the inner boundary layer, the other part reuniting



with the middle layer. On the ventral side of the adoral zone the reflected layer turns immediately caudad. Part of it fuses with the inner boundary layer, though at a point farther caudad. Dorsally, ventrally, and laterally, part of the reflected middle layer joins the inner layer and will be followed in the description of the inner layer (text figs. 1, 2, and 3). The reflected middle layer appears, in longitudinal sections through the dorsal side of the adoral zone, as a body which greatly resembles the motorium of Sharp ('14) (text figs. 1 and 3). In cross-sections (text fig. 2, sections A and B) this layer is seen to be reflected over the anterior end of the skeletal structure. It is this relationship with the skeletal laminae that makes it difficult in longitudinal sections to see the two layers which constitute this fold.

The fibers which Sharp ('14) described as connecting this body with the esophagus and with the membranelles of locomotion and of food ingestion could not be differentiated. Attention is called to plate 1, figure 4, which is a photomicrograph of *D. ecaudatum*. A fiber can be seen which resembles the fiber designated as the esophageal ring in Sharp's ('14) photograph, plate 1, figure 4. However, in the present writer's material this ring is a section of the inner boundary layer of the ectoplasm—hence the conclusion previously published (Rees, '30) that Sharp misinterpreted a fold of the middle ectoplasmic layer as a motorium and sections through this layer as strands.

#### THE INNER BOUNDARY LAYER

It is well to recall that the living ciliate may, in response to the proper stimulus, retract the entire anterior end of the body, which contains the oral and the dorsal membranelle zones, within the more rigid part of the ectoplasm. This retraction greatly alters the relationships of the anterior structures by eliminating the folds which produce the various lips and furrows, but it alters the shape and position of the inner boundary layer only in the zone around the esophagus. This layer, therefore, may be considered as a bag containing the fluid endoplasm.

However, as has been previously indicated, this bag has two openings, one for the esophagus and one for the cecum. The pellicle forms the lining of the esophagus, and the walls of this tube are formed by the fused middle and inner ectoplasmic layers (text fig. 3, sections C and D, and pl. 2, figs. 7 to 11). On the dorsal side of the esophagus the relationships are more complicated than on the ventral side. It appears that sheets of this fused layer (shown in sections as strands) branch off and extend diagonally cephalad and are reflected back along the esophagus (text fig. 1 and text fig. 2, section D). Further complications occur near the macronucleus and the micronucleus, which, while wholly within the ectoplasm, are invested by a specialized layer which likewise appears to be connected to the inner boundary layer of the ectoplasm (text fig. 1 and text fig. 3, sections D and E). However, dorsally and ventrally, as well as laterally (text figs. 1, 2, and 3, and pl. 2, figs. 7 to 11), the esophageal walls are formed by a cylindrical structure which is composed of these two ectoplasmic layers. At the caudal end of the body the relationships of these layers appear to be fundamentally the same as at the anterior end, though much simpler than at the latter end because there are few folds and no membranelles. It is shown in text figure 1 that the walls of the cytoppyge, the rectum, and the cecum, like those of the esophagus, are formed by the two ectoplasmic layers. However, in the walls of the cecum the two layers are seen to be continuous like the walls at the mouth of a thermos bottle. This relationship suggests that the two ectoplasmic layers form a double-walled sac about the endoplasm, as shown in text figure 6. According to this concept, only the pellicle is broken through in the esophagus and in the cecum, while the middle and the inner ectoplasmic layers are continuous; their relationship is somewhat the same as that of the splanchnic and of the somatic mesoderm of metazoan embryos.

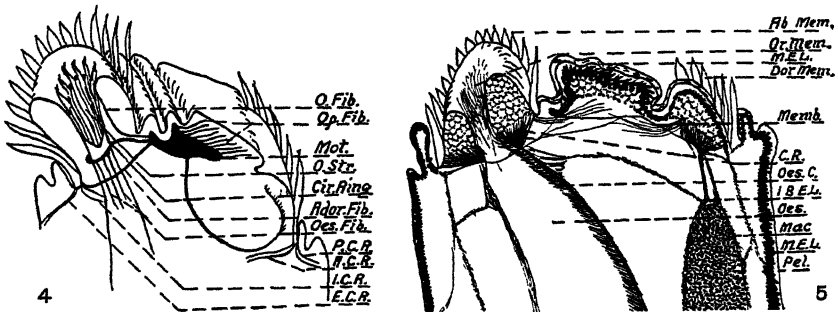
*1. The fibrillar systems of the middle and the inner ectoplasmic layers*

Sharp ('14) described the inner boundary layer of the ectoplasm as a "structureless membrane separating the ectoplasm from the endoplasm and bounded on each side by a layer of alveoli." The present writer found, in disagreement with Sharp ('14) and in agreement with Eberlein ('95), that this layer has a definite fibrillar structure, and also in agreement with Gunther ('00), that alveolar layers are absent. In text figure 7 the writer has attempted to illustrate this interwoven network in greater detail than is shown by the photomicrograph (pl. 1, fig. 6). In *D. medium* and in *D. maggii* the fibrillae may be differentiated with especial clearness, particularly in sections 3  $\mu$  thick (text fig. 5 and pl. 1, figs. 1, 5, and 6, and pl. 2, figs. 7 to 13). They are difficult to demonstrate in *D. ecaudatum*. The occurrence of fine fibrillae in the non-ciliated ectoplasm of *Diplodinium* is of interest in connection with other papers on neuromotor systems. For example, the writer ('22), Lynch ('29, '30), and others have demonstrated the occurrence in various ciliates of systems of fine fibrillae which appear to be connected on the one hand to the cilia and on the other to a coordinating center. This work should be carefully reviewed in the light of the above findings for *Diplodinium*. It is obvious that in the latter ciliate the fibrillae of the ectoplasmic layers have no relationship to a neuromotor system.

THE CILIA AND THE CILIARY ROOTLETS

Text figure 3, section F, shows each cilium connected to a basal granule to which the proximal ends of the ciliary rootlets also are attached. In other sections the cilia appeared to be connected to a basement membrane; it was not possible to distinguish individual granules. However, thin sections, carefully destained, show the individual granules which also serve as the proximal attachments of the ciliary rootlets. The distal attachments of these rootlets are to one or the other of two membranes (text figs. 1, 3, and 5, and pl. 2, figs.

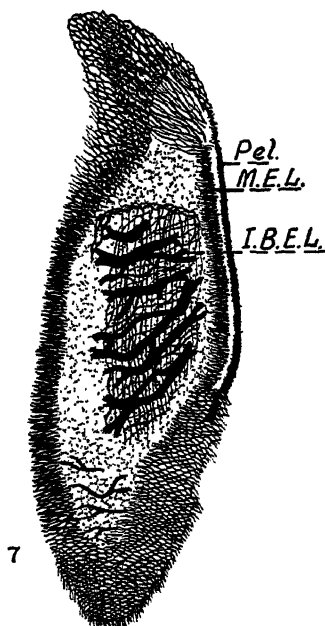
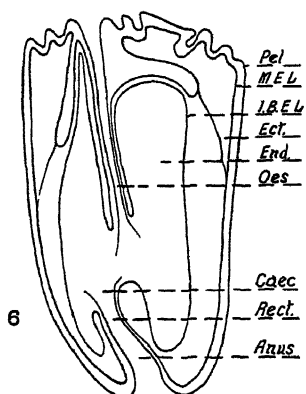
7 to 13). On the ventral side of the body one of these membranes begins near the base of the inner adoral lip and appears to be attached to the middle layer of the ectoplasm. From this point it extends transversely, being pierced by the esophagus, and divides into three strands, each of which appears to be connected to a second membrane. The latter membrane extends diagonally and caudally from the mass which resembles Sharp's motorium to the dorsal side of the



Text fig. 4 Diagram, modified after Sharp, of *Diplodinium ecaudatum* to show the neuromotor apparatus. *O.Fib.*, oral fiber; *Op.Fib.*, opercular fibers; *Mot.*, motorium; *O.Str.*, esophageal retractor strands; *Cir.Ring.*, circumesophageal ring; *Ador.Fib.*, adoral fiber; *Oes.Fib.*, esophageal neuromotor fibers; *P.C.R.*, *A.C.R.*, *I.C.R.*, and *E.C.R.*, the posterior, anterior, internal, and external ciliary rootlets.

Text fig. 5 Diagram of *Diplodinium medium*, to be compared with text figure 4 of *D. ecaudatum*. *Ad.Mem.*, adoral membranelles; *Or.Mem.*, oral membranelles; *M.E.L.*, middle layer of the ectoplasm; *Dor.Mem.*, dorsal membranelles; *Memb.*, membrane; *C.R.*, ciliary rootlets; *Oes.C.*, esophageal cilia or membranelles; *Oes.*, esophagus; *Mac.*, macronucleus; *Pel.*, pellicle.

body, at which point it is attached to the middle layer of the ectoplasm. Further reference to text figures 1 and 3 shows that for many of the ciliary rootlets a distal attachment could not be found, notably, 1) the fibers which Sharp ('14) called anterior ciliary rootlets extending diagonally and ventrally from some of the dorsal cilia to a zone beneath the operculum, and, 2) the internal ciliary rootlets which Sharp ('14) found extending diagonally in the opposite direction from the adoral membranelles and terminating in the same opercular zone.



Text fig. 6 Diagram of *Diplodinium medium*, showing the probable interrelationships of the various layers of the ectoplasm. *Pel.*, pellicle; *M.E.L.*, middle layer of the ectoplasm; *I.B.E.L.*, inner boundary layer; *Ect.*, ectoplasm; *End.*, endoplasm; *Oes.*, esophagus; *Caec.*, cecum; *Rect.*, rectum; *Anus.*, anus.

Text fig. 7 Camera-lucida drawing of section no. 3 of a series that contained forty-two longitudinal sections of  $3\mu$  through *Diplodinium medium* ( $\times 600$ ), to show the complex network of interwoven fibers of the middle layer and of the inner boundary layer of the ectoplasm. Abbreviations as in text figure 1.

Reference to text figure 4, which is a figure of *D. ecaudatum* modified after Sharp, shows four sets of ciliary rootlets, viz., the anterior and the internal mentioned above, and also the posterior and the external. A comparison of text figure 4 of *D. ecaudatum* with text figure 5 of *D. medium* shows a general agreement between Sharp's account and the writer's account of the ciliary rootlets. However, so far as can be ascertained from his paper, Sharp did not regard these ciliary rootlets as a part of the neuromotor system. Furthermore, he apparently did not find either of the two membranes mentioned above. The following evidence is cited to show that they are membranes rather than fibers:

One or the other of them occurred in all longitudinal and oblique sections, whether cut with reference to the parasagittal plane or to a plane at a right angle or at any other angle to it. The present writer's figures suggest that the mechanism of *D. medium*, in which the ciliary rootlets are attached to membranes which in turn are attached to the fibrillar systems of the ectoplasmic layers, appears adapted to function better in the retraction of the adoral and of the dorsal cilia than in the coordination of the movements of these organelles.

#### THE ESOPHAGEAL MEMBRANELLE ZONE

Sharp ('14) suggested that the oral cilia of *D. ecaudatum*, the food of which ciliate was said to consist entirely of bacteria, may extend partway down the esophagus, but he made no reference to esophageal membranelles. A detailed description of the mechanism which enables free-living ciliates to ingest bacteria has been worked out, particularly in *Paramecium*, by Nirenstein ('05), Rees ('22), and others. In *Paramecium* the cytopharyngeal cilia carry the ingested bacteria to the distal end of the esophagus, where they are forced into food vacuoles which are finally pinched off to circulate in the endoplasm until intracellular digestion is accomplished. The present writer ('30) was unable to understand from Sharp's account how the bacteria could be forced by the oral cilia only through the long esophagus. However, at that time

the writer's sections did not show any esophageal membranelles. These membranelles were first seen in cross-sections of individuals that were embedded by Zirkle's ('30) method from N-butyl alcohol. Comparison of text figure 1, and text figure 2, sections D and E, and text figure 3, sections B and C, which are of *D. medium*, with sections of *Paramecium* in the paper by Rees ('22) shows that in the former ciliate the esophageal cilia are rather feeble. This fact probably accounts for the general failure to find them. However, Awerinzew and Mutafova ('14) illustrated two prominent membranelle zones on each side of the esophagus of *D. medium*, but their figure does not appear to be based on careful studies of sections. Three probable explanations are here proposed for the relatively feeble development of esophageal cilia in the genus *Diplodinium*. 1) The bacterial flora of the rumen and the reticulum being very abundant, the necessary food may be ingested without much effort; 2) other substances such as fragments of hay and grass, for the ingestion of which the esophageal cilia are not required, may serve as food; and, 3) these ciliates, by capturing and ingesting smaller ciliates, may have adapted themselves to a carnivorous diet. The writer's studies of these possibilities were conducted with fixed material only; further research should be conducted with living organisms.

#### THE ESOPHAGEAL RETRACTOR STRANDS

Sharp ('14) suggested that retraction of the oral region is accomplished by a set of fibers, the esophageal retractor strands, the insertion of which was stated to be at the oral end of the esophagus and the origin in the skeletal structures, at the caudal end of the body. However, it has been shown in the preceding paragraphs that in *D. medium* the esophageal fibrillae are part of an extensive fibrillar system which embraces the entire middle and the inner layers of the ectoplasm. Further research is needed to determine how retraction of the oral region is accomplished.

## DISCUSSION

According to Sharp ('14), the neuromotor system of *D. ecaudatum* is constituted as follows: 1) A motorium or neuromotor center; 2) an esophageal fiber connecting the motorium to the esophageal ring; 3) a fiber leading from the motorium to the adoral ciliary zone; 4) another fiber from the same body to the oral zone; 5) a fourth fiber to the dorsal ciliary zone; and, 6) many fibers, for which Sharp suggested a sensory function, leading from the motorium to the outer ectoplasm beneath the operculum (text fig. 4). The writer's diagram of *D. medium* (text fig. 5) shows a fold of the middle layer of the ectoplasm, which corresponds in its position to Sharp's motorium, and also numerous fibers corresponding to the opercular fibers. However, in other respects the writer's account is wholly at variance with that of Sharp. On the other hand, it has been discovered that the ciliary systems of *D. medium* and of *Paramecium ecaudatum* are remarkably similar. In both of these species the esophagus is provided with cilia; and all of the cilia of the body appear to have ciliary rootlets. In *Paramecium ecaudatum* these rootlets appear to converge to a point near the esophagus. In *Diplodinium medium* this convergence is not so manifest, but the connection of many of the rootlets with a membrane suggests a similar relationship. The only plausible explanation for this striking similarity in distantly related organisms is that of descent from a common ancestor. On the other hand, the discrepancies mentioned above in closely related species between Sharp's account of *D. ecaudatum* and the present writer's account of *D. medium* are out of harmony with the above generalization. These discrepancies are not confined to the neuromotor system, because in *D. ecaudatum* one of the ectoplasmic layers was said to be structureless and the other alveolar, whereas in *D. medium* they have both been shown to be fibrillar. Furthermore, a system of esophageal cilia has been demonstrated in *D. medium*, which system should be looked for in *D. ecaudatum*, especially in view of Sharp's statement that the food of the latter ciliate consists



entirely of bacteria. It is, therefore, suggested that further research be conducted with these two species and with other species of the genus. For this work the present writer has contributed a new method of approach, viz., the sectioning of individuals rather than of masses of ciliates.

#### SUMMARY AND CONCLUSIONS

1. About eighty ciliates of the species *Diplodinium ecaudatum*, *D. maggii*, and *D. medium* have been isolated with a micropipette, embedded in paraffin, and cut into serial cross and longitudinal sections of  $3\ \mu$  so that the ectoplasmic layers could be followed as are the germ layers in metazoan embryos.

2. The method of Zirkle ('30) of embedding plant tissue from 100 per cent N-butyl alcohol into paraffin has been used for ciliates with success, and found superior to previously used methods.

3. It is suggested that the motorium described by Sharp ('14) is a fold of the middle layer of the ectoplasm, and that this body does not appear to function as a coordinating center of a neuromotor mechanism.

4. The fibrillar system described and figured by Sharp ('14) as connecting the motorium to the membranelles of locomotion and of food taking and to a ring around the esophagus could not be differentiated.

5. The ectoplasmic layers of *D. medium* are made up of a complex system of interwoven fibrillae.

6. It is suggested that in *D. ecaudatum* as in *D. medium* the esophageal retractor strands are part of the above interwoven network.

7. In *D. medium* delicate membranes have been discovered at the anterior end of the body to which many of the ciliary rootlets are attached at their distal extremities. These membranes are connected to each other and to the middle layer of the ectoplasm and are believed to function in the retraction of the adoral and the dorsal cilia.

8. A system of esophageal membranelles has been described in *D. medium* which serves to explain the method of ingesting bacteria.

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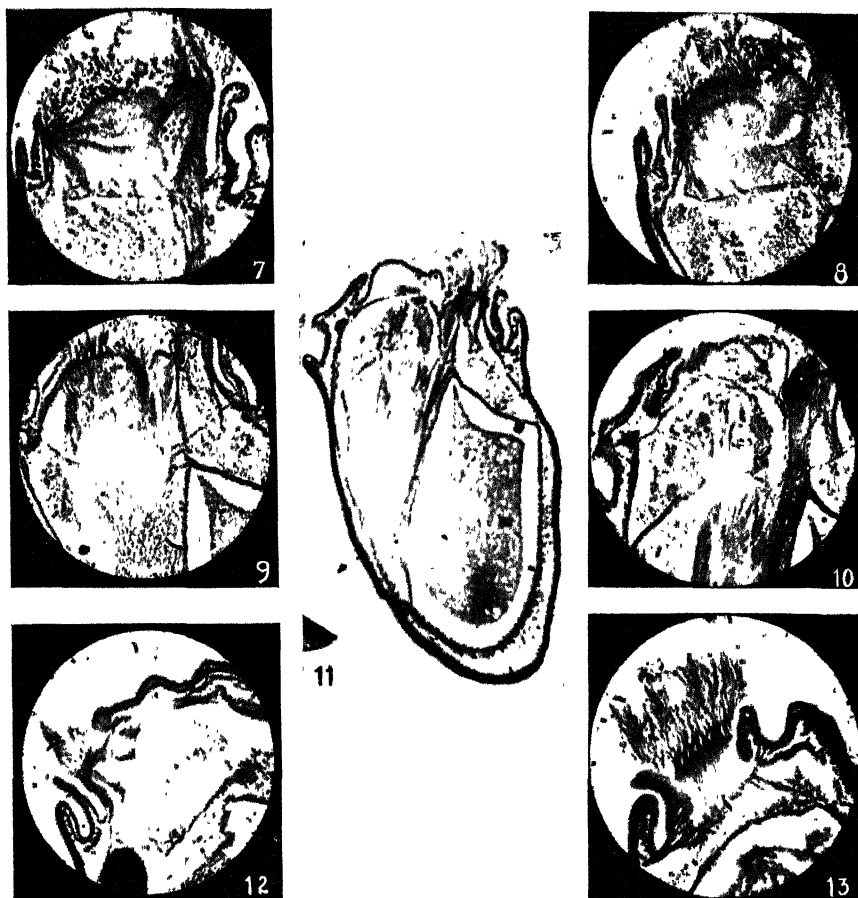


Photomicrographs of longitudinal sections,  $\times 200$ , of three species of *Diplo-dinium* stained with iron haematoxylin.

1 and 2 *D. maggii* at  $3\mu$ ; note the motorium-like structure in figure 1; this structure occurs in the non-ciliated portion of the ectoplasm, and is, therefore, not associated with a neuromotor system.

3 and 4 *D. caudatum* at  $5\mu$ ; note the structure in figure 3 which resembles part of an esophageal ring; it is a section through a part of the inner boundary layer of the ectoplasm.

5 and 6 *D. medium* at  $3\mu$ ; note the complex interwoven network of fibers in the ectoplasm.



Photomicrographs of six longitudinal sections of *Diplodinium medium* of the same series as was selected for the drawings of text figure 3. Figures 7, 8, 9, 10, 12, and 13, about  $\times 750$ ; the magnification of figure 11 was not determined.

7 Serial section no. 8; compare with text figure 3, section A; note the relationship of the inner boundary layer to the walls of the esophagus; note also the transverse membrane.

8 Serial section no. 10.

9 Serial section no. 12; compare with text figure 3, section B; the walls of the esophagus are shown also in these figures to be formed of the inner and of the middle layers of the ectoplasm.

10 and 11 Serial section no. 16; compare with figure 3, section C.

12 Serial section no. 23; compare with figure 3, sections D and E; note that the ectoplasmic layer which invests the macronucleus is connected to the membrane.

13 Serial section no. 26; compare with figure 3, section F.



# THE DEVELOPMENT OF THE CARP, CYPRINUS CARPIO

## I. THE LARVAL LIFE OF THE CARP, WITH SPECIAL REFERENCE TO THE DEVELOPMENT OF THE INTESTINAL CANAL

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### SEVEN FIGURES

#### AUTHORS' ABSTRACT

The detailed spawning habits and hatching period are described. The larval period is divided into the inactive, when the yolk is the source of food, and the active period, when food is captured. The rate of growth and transformation of the larvae into fry is described.

The cell formation passes into a syncytial period, when there are produced many nuclei without cytoplasmic division. Around these nuclei, the cytoplasm collects to give rise to more cells. The method by which mucous cells are produced is described and also the presence of secretion masses in both the mucous cells and the syncytium of the mucosa.

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### INTRODUCTION

The carp was introduced into the fresh waters of the United States about one hundred years ago. During this period it has become generally distributed, at first through the agency of the United States Bureau of Fisheries, but mostly by reason of its own adaptive habits.

These adaptive habits of the carp consist in their ability to thrive in streams polluted by sewage or in relatively pure lake waters; in their omnivorous food habits; in the great

variety of places and seasonal periods when their vast number of eggs are laid; and in their habits during the larval period and early fry stages.<sup>1</sup>

So successful have carp become that their very numbers are regarded as a menace to the development of the more eagerly sought food and game fish. This condition has stimulated the Conservation Department in several states to undertake extensive field studies. It became evident very soon that no control measures could be formulated until the biology not only of our fresh waters was better understood, but what was more important, the biological factors that were involved in the habits of an old and generalized fish like the carp. In order to establish some of these fundamental facts, a series of studies has been planned that it is expected will furnish the basis for control measures.

#### SPAWNING PERIOD

The carp usually come into shallow water to spawn and the time of spawning in the spring seems to depend on the temperature of the water. In Oneida Lake there is often a close association between that time and the period when chara and potamogetons have reached a size of about 1 foot in height. One season this was as early as July 5th, and the following year, July 23rd; but in the year when spawning was so late in Oneida Lake, the carp spawned in swamps in April. During the past summer, Professor Struthers observed them spawning in August. While there is some evidence in support of the idea that there is no fixed spawning period, it is certain that eggs are very extensively laid in the spring and early summer. The question of whether the same fish spawns more than once in a single season has not been answered. We are inclined to doubt if carp spawn twice in a season and suggest

<sup>1</sup> W. M. Smallwood and Parke H. Struthers. 1928. Carp control studies in Oneida Lake. New York Conservation Commission.

Parke H. Struthers. 1927, 1928, 1930. Carp control studies. New York Commission.

W. M. Smallwood and Mary L. Smallwood. 1929. The German carp, an invited immigrant. *Scient. Mont.*, vol. 29.

that summer and early fall spawning is dependent on the time when the eggs in the female reach maturity. The chief facts in support of this suggestion are the wide range of size<sup>2</sup> in carp of the same age. One lot of carp were hatched this past summer and retained in the same aquarium. These were regularly fed on daphnia and in a month some of the fry were twice the size of the smaller ones.

#### HATCHING PERIOD

The eggs of the carp have been repeatedly collected and carp have been observed by the senior writer to spawn so close to where he was standing that, had he moved, the fish could have been touched. Some of these eggs were collected and placed in various-sized dishes. If the water is warm, as when the dishes are set in a window where the sun shines, the embryos begin to show movements on the third day. Active movements are characteristic on the fourth day. By the fifth day some may have escaped. By the end of the seventh day, practically all of the eggs have hatched. But when jars are kept in the shade where it is cool, the hatching period is delayed. In every batch collected some of the eggs were apparently unfertilized, and in some this represented a large proportion, as if the female had spawned alone. When the eggs were placed in bass cages of fine-mesh wire, 3 feet in diameter, in the lake, most of the eggs failed to hatch. This was probably due to the fact that the waves washed mud into these cages, for on the second day they were covered with saprolegnia. Our observations on the places where eggs were laid show that they were protected from the mud due to waves or attached to plants, so that they were continually aerated. In some places the mortality of the eggs is very great.

#### THE LARVAL PERIODS<sup>3</sup>

The importance of a study of the larval stages of fishes has long been realized, yet no really accurate work had been done

<sup>2</sup> See previous references.

<sup>3</sup> The remainder of this paper is largely condensed from the master's thesis of the junior author.



before 1915. Kuntz and Radcliffe<sup>4</sup> state that "adequate measures for conservation of our fishery resources—require as their basis a reasonably complete knowledge of the life histories and habits of fishes." Welsh and Breder<sup>5</sup> are more emphatic in their assertion that "A fundamental prerequisite for intelligent fisheries legislation is an accurate knowledge of the life histories of the species contributing to that supply."

The larval stages<sup>6</sup> of the carp fall into two periods, the inactive period, when the yolk supply is still adequate, and the active period, when important morphological changes take place.

#### *Inactive larva*

When the carp first hatches, there is a conspicuous amount of yolk that is crowded nearly around the anterior end of the intestine and extends almost to the vent (fig. 1). This period lasts from three to four days, depending on temperature. The specimen in figure 1 was killed eight days after the eggs were laid. The head is small and the jaws and snout feebly developed. The dorsal fin is continuous around the tail to the anal opening. There is no evidence of the paired pelvic and pectoral fins, while the caudal fin is of the primitive protocercal type in which the notochord is straight. A few rays begin to appear in the caudal fin. The forming air bladder pushes the otherwise straight intestine ventrally. A large ear vesicle is evident. Pigment exists chiefly along the lateral-line region and is scattered over the head. Each pigment cell is branched at this stage.

<sup>4</sup>Kuntz, A., and Radcliffe, L. 1915-1916 Notes on the embryology and larval development of twelve teleostean fishes. Bulletin of the Bureau of Fisheries, vol. 35, document no. 849.

<sup>5</sup>Welsh, W. W., and Breder, C. M., Jr. 1923-1924. Contributions to life histories of Sciaenidae of the eastern United States coast. Bulletin of the Bureau of Fisheries, vol. 39, document no. 945, pp. 141-143.

<sup>6</sup>Stewart, Norman Hamilton. Development, growth and food habits of the white sucker, *Catostomus commersonii* Lesueur. Department of Commerce Bulletin, Bureau of Fisheries document no. 1007.

*Active larva*

During the first three or four days after hatching, the larval carp remain attached to the side of the dish or to plants. When touched, they swim about for a few seconds, then come to rest. But by the end of this period there is voluntary activity and the larva has begun its career and must capture its own food.

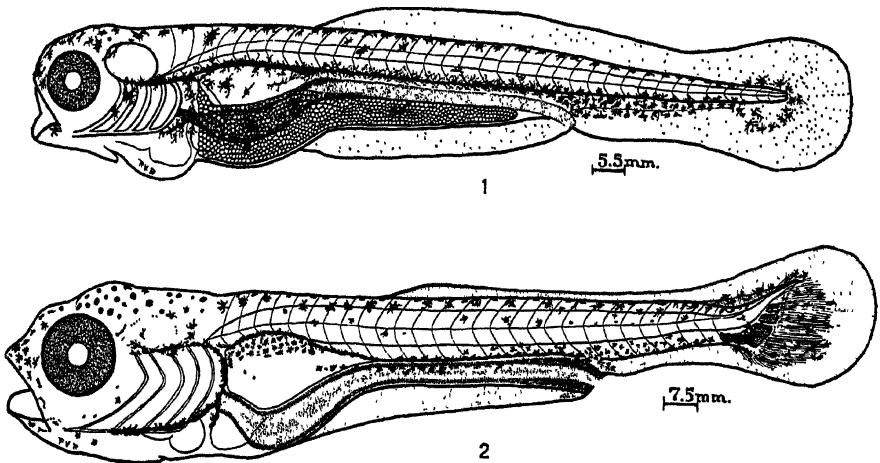


Fig. 1 Inactive larval stage of carp 5.5 mm. long. Sketched by Phyllis Plyler. The following measurements will serve to show the relative bodily changes in comparison with the active larva, figure 2. Eye diameter, 0.34 mm.; depth of head through eye, 1 mm.; heart, 0.13 mm.; ear,  $0.15 \times 0.30$  mm.; ventral fin, 1.60 mm.; dorsal fin, 2.16 mm.; length of intestine, 1.80 mm.

Fig. 2 Active larval stage of carp 7.5 mm. long. Sketched by Phyllis Plyler. Eye diameter, 0.60 mm.; depth of head through eye, 1.25 mm.; heart, 0.4 mm.; ear,  $0.30 \times 0.55$  mm.; ventral fin, 2.10 mm.; dorsal fin, 2.60 mm.; length of intestine, 3 mm.

In figure 2 the early period of the active larva shows that the head has noticeably changed its shape. The jaws are larger, as is the whole head. Yolk is no longer present and the increase in the air bladder emphasizes the stomach-like bend in the intestine. The dorsal fin is being absorbed, except at the anterior end. Rays are numerous in the caudal fin, which is now heterocercal, having the notochord extending dorsally. Pigmentation is more general and some of the

pigment cells are no longer branching. It is difficult to state just when the larval stage ends, but in two weeks the body takes on the characteristic hump just back of the head, the fins have become formed, and the pigmentation begins to have the golden tints that are so characteristic of the fry stages.<sup>7</sup>

The young carp are very active feeders within their local habitats and grow rapidly, as the following indicates:

	<i>Length, mm.</i>		<i>Length, mm.</i>
July 12,	10	August 12,	38- 42
July 21,	12-26	August 20,	41- 78
July 27,	14-30	September 5,	80-112
July 29,	12-40	September 7,	72-106

#### THE GROWTH OF THE INTESTINE

The duct from the liver enters the intestine close to the pharynx, the esophagus emptying directly into the intestine. Just before hatching, the simple tube of columnar epithelium is completed. The chief modification in this simple tube during the larval periods is the ventral bending in the anterior end as the air bladder grows. The simple, relatively straight tube persists until the young fry stage. In the fry stage there is an active growth in the intestine that continues for at least two years.

The specific length of the young carp is not an indication of the degree of intestinal coiling, for one specimen 1½ inches long had one small loop, while another of the same length had more than doubled the length of the intestine (fig. 3, A). Young carp taken in early fall are about 3 inches long. By this time the intestine is coiling in the most anterior loops (fig. 3, B). A carp 13½ inches long is between one and two years old, provided it has lived in non-polluted waters where food was abundant. By this time the coiling has become extensive and irregular, as there is no uniformity or order in the formations of the coiling (fig. 3, C).

The anterior limb of the intestine as well as the rectal portion remains straight. The anterior portion has a greater

<sup>7</sup> See colored plate of carp fry in *Carp control studies, 1927*, Smallwood and Struthers.

diameter and slightly firmer wall. This superficial resemblance to a stomach has led to an error in stomach-content studies. What the several investigators have done is to record the contents of this first section of the intestine, which is not separated from the remainder of the digestive tract by any morphological features.

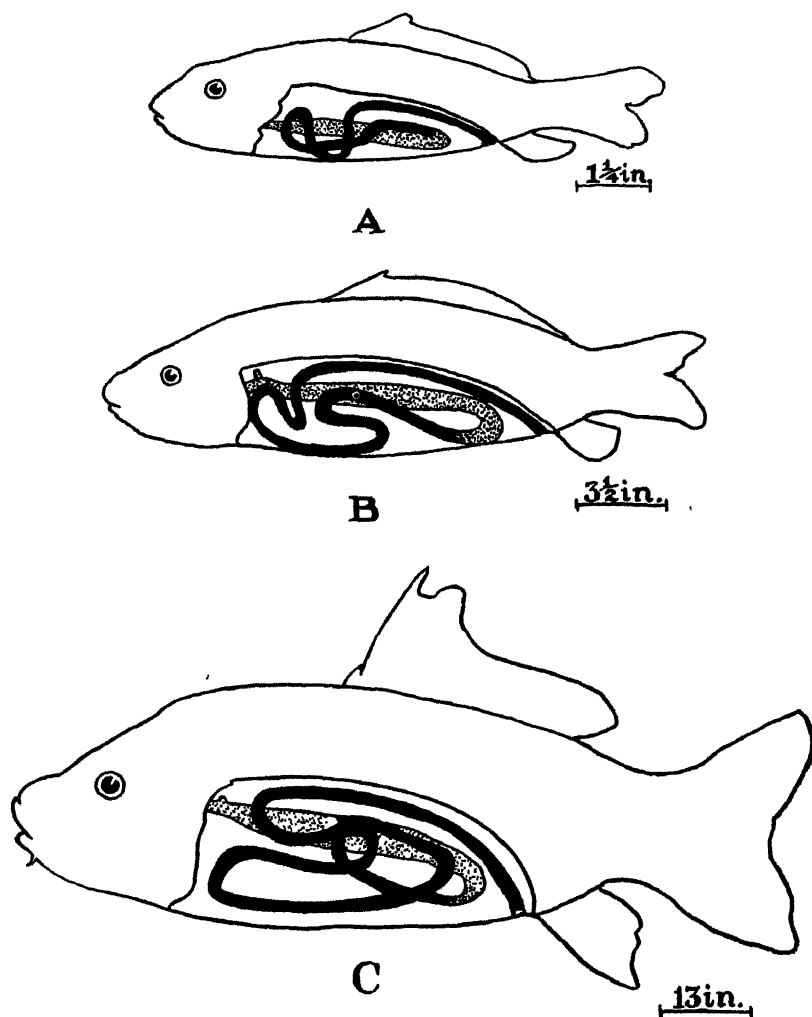


Fig. 3 Diagrammatic sketches to show the manner of folding of the intestine and the extent of growth during the first year and a half.

During this period, when the intestine is undergoing this great extension in length, the carp takes on its habit of feeding on vegetal débris, and there seems to be a close relation between this adaptive habit and the extensively developed intestine when compared with the carnivorous feeding larva or the fish that subsist on minnows. It is in this very fundamental structure that we have one of the explanations for the ability of the carp to subsist on a widely varied diet—and, because of its basal nature, one of the most difficult to control.

The intestine of a carp 24 inches long has reached a length of about 40 inches and has a diameter of 2 cm. When the intestine first forms, just before hatching, there is a single row of columnar cells enclosing a minute cavity. Twenty cells are sufficient to encompass such a stage, while at least 500 are required in a 15-mm. larva having an intestine 1 mm. in diameter. As there is very little change in the diameter of the mucous cells, we would have many thousand cells required in the adult if there were no folds. Inasmuch as there is a very extensive folding, the approximate number of cells in a single row in the adult must be very great. When the great increase in length is considered, the total number of mucous cells that must form is very large.

The first change consists in the increase in number of the simple columnar cells, resulting in a simple straight tube surrounding a well-defined lumen (fig. 1). By the time that the active larval period is reached, minute folds begin to form in this simple mucosa, progressing from the anterior end posteriorly. During the same period, digestion is taking place in the active larva and the young fry.

#### MODIFICATIONS IN THE MUCOSA

In certain regions we find mitotic figures, some located close to the basement membrane and others near the lumen of the intestine. The position of the mitotic figure does not correspond to the axis of the simple columnar cells (fig. 4, A, B). As the division of cells continues, nests of nuclei are formed. The cell walls gradually disappear, resulting in a syncytium (figs. 4, B; 5, A, B).

In the earlier stages of the active larva (figs. 4, 5, 6) the nuclei tend to collect near the basement membrane, after which cell walls can faintly be recognized (fig. 6). One would expect to find all stages somewhere in the length of the intestine of a single animal, but what really happens is that nearly

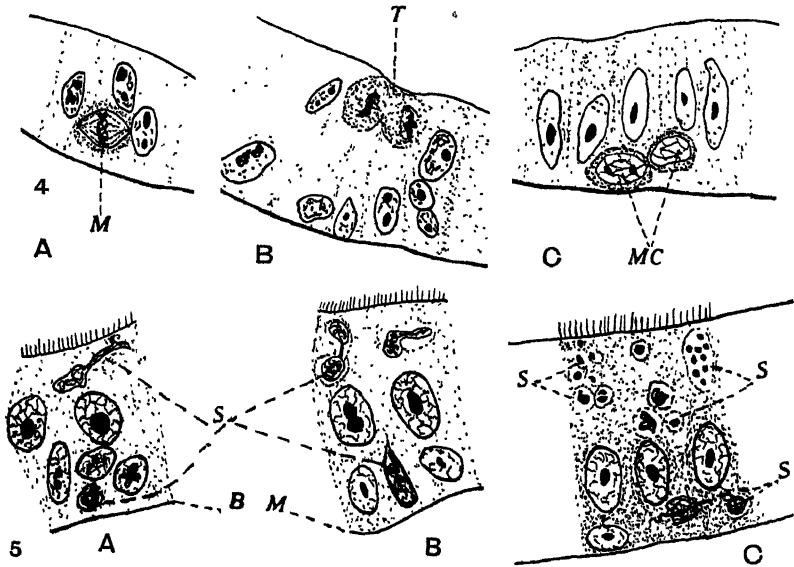


Fig. 4 A. Mitotic figure close to basement membrane in late inactive larva. B. Late telophase with direction of division across the cells of the mucosa and close to the distal end of the mucosa. Nuclei are beginning to form a nest close to basement membrane. C. An early stage in the formation of mucous cell, *m.c.* Drawn with Zeiss 2-mm. oil-immersion 10 eyepiece and camera lucida.

Fig. 5 Synektial condition of the mucosa in active larval stage. A. Large secretion mass, *s*, near distal end of mucosa. Close to basement membrane, *b.m.*, is a small secretion mass. B. The secretion masses in distal region are in vacuoles. C. The secretion masses have broken up into minute spherical bodies preparatory to passing into the lumen of the intestine. Drawn with Zeiss 2-mm. oil-immersion 10 eyepiece and camera lucida.

the entire length of the mucosa has the cells in the 'resting stage.' The steps shown in figure 4, A, were drawn from one animal and those in figure 5, A, from another.

After a period of mitotic activity in some specific region, the cytoplasm becomes organized around these nuclei, as in figures 4, C, and 6, A. The process of enlarging the diameter

of the intestine and forming simple folds in the intestine of the active larva is relatively simple. In the young fry stage, the process, while simpler, involves a greater number of cells and the thickness of the mucosa is greater.

The conditions shown in figure 6, B and C, were drawn under the oil immersion and with the camera lucida, without moving the section. The distance between these two conditions was but a half-dozen cells in width. Cells such as those in figure 6, B, covered the fold, while just at the base of the

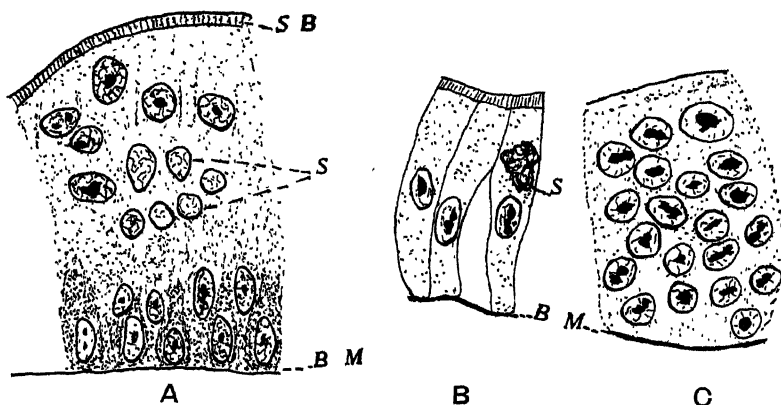


Fig. 6 A. The striate border, *s.b.*, is distinct in this synectium and the definitive cells of the mucosa are forming close to the basement membrane. Secretion masses, *s*, are seen in an early stage. B. Character of the mucosa cells in a 15-mm. larva. *s*, secretion mass. C. A characteristic nest of nuclei that extended for  $30\mu$  in length in a 15-mm. larva. Drawn with Zeiss 2-mm. oil-immersion 10 eyepiece and camera lucida.

fold, there was this nest of nuclei extending from the basement membrane nearly to the striate border. Many of these nuclei were in pairs, showing that they had recently been formed. In this same section mitotic figures were present, indicating the process of multiplication. There is no evidence of cell walls in figure 6, C.

It may possibly be objected that the masses of nuclei are leucocytes. In order to settle the question as to the nature of the secretion masses, the intestine was fixed in Zenker-formol fixative, and Maximow's modification of Giemsa's

stain was used. This stain is selective for the leucocytes and eosinophils. When this technique is used, there can be observed in the submucosa vast numbers of eosinophils and a few leucocytes having the cytoplasm stained blue. The mucosa, however, is almost free of each of these blood corpuscles. A few leucocytes can be identified and an occasional eosinophil. The structure of the nucleus of the mucosa is so characteristic that one would not confuse it with the nucleus of any of the white blood corpuscles. The blood of the carp is being studied by Doctors Lindeman and Robeson.

It is difficult to discover any measure for the period of time that is required for these changes. They occur in specific and limited regions in which only a small portion of the total number of cells in a given section participate. The sequence of events in the transformation of such a nest of nuclei into an epithelial mucosa is similar in all instances. The cytoplasm takes a deeper stain close to the nuclei, which are arranged in irregular rows near the basement membrane. This reorganization of a tissue out of a syncytium takes place gradually, for one will find but very few cells forming at one place, and at another the number of basal nuclei is limited to clusters of two's, three's, or four's.

In longitudinal sections of the intestine of fry four months old, one usually encounters syncytial masses of nuclei at the bends of the intestine (fig. 3) and in the mucosa between the numerous folds. This suggests that there are active regions of growth in the mucosa which for a time are given over to cell formation.

#### FORMATION OF MUCOUS CELLS

Some of the basal nuclei contribute directly to the formation of the mucous cells. The cytoplasm collects about such nuclei (fig. 4, C, *m.c.*) and then gradually extends toward the lumen of the intestine. The distal end becomes transparent even before the proximal end is in contact with the basement membrane (fig. 7, B, *m.c.*). These steps are more easily observed in the late inactive larva. Even at this early stage



irregular line-like bodies can be observed in the distal region. These same bodies are very clearly shown by Maximow's stain and are similar to the mitochondria which Maximow describes for the mucous cells. These same mitochondria-like bodies are present in the adult mucous cells (fig. 7, A).

#### DIGESTION IN THE LARVA AND FRY

Such animals as the carp solve the problem of digestion by an intensive development of intestinal secretion. One must keep in mind that there are no gastric glands, although the character of the folds often simulates stomach conditions, and that the duct from the combined liver and pancreas

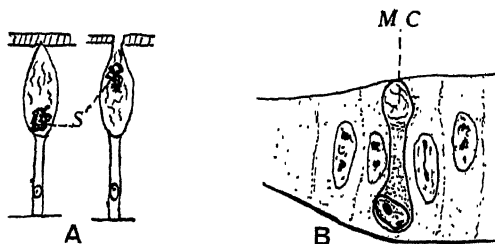


Fig. 7 A. The adult mucous cell, showing mitochondria bodies and a secretion mass, *s*. B. The formation of the mucous cell, *m.c.* Drawn with Zeiss 2-mm. oil-immersion 10 eyepiece and camera lucida.

enters the most anterior portion of the intestine. This same combined digestive gland is small in the larva and young fry, and the cells are in active growth, the details of which will form a separate paper. In the early larval stage carp begin capturing cyclops and small daphnia. The entire length of the intestine may be packed with these minute crustaceans. As one observes the condition of the organs in these ingested animals, all of the flesh is dissolved in those found in the posterior region except the eggs in the egg case of daphnia. This resists the digestive action and one can observe the eggs in various stages of segmentation. There can be no question but that the flesh of these crustaceans is dissolved as the ingested animals move along the intestinal passage. The minuteness of the liver—the pancreas has not yet formed—

practically eliminates this organ from participating in the digestion of these crustaceans.

While trying to analyze the nature of cell formation in the mucosa, our attention was attracted to a number of irregular bodies that stained conspicuously with iron-haematoxylin and looked very much like a nucleus in the spireme stage (figs. 5, A; 6, B, *s*). But when one was found that extended across several cells in width (fig. 5, A, B, *s*), it was clear that the structure could not be classified as a nucleus. These masses were found to form near the basement membrane and gradually move toward the lumen. As they enter the distal portion of the mucosa, a clear area surrounds them. These large masses now fragment (fig. 5, C, *s*) and the network-like body transforms into a series of minute spherical bodies that pass into the lumen of the intestine.

These bodies are interpreted as secretion masses. Some evidence of Golgi bodies was found in connection with the nucleus, but no definite connection could be observed between them and these secretion masses. Their staining reaction with iron-haematoxylin would be sufficient to render them distinct from the Golgi bodies. It is clearly evident that the syncytial mucosa forms these secretion masses and that in it they are the same as the phenomenon that takes place in cells (figs. 5, A, B, C, and 6, B). In figure 6, A, there are secretion bodies that take a very faint stain. These bodies might be mistaken for newly formed nuclei except that they lack the characteristic chromatin mass (fig. 6, C). The secretion bodies in figure 6, A, *s*, are interpreted as an early stage in their formation which is followed by the conditions illustrated in figures 6, B, *s*, and 5, A, B, *s*. The final changes are shown in figure 5, C, *s*.

The conspicuousness and number of these secretion masses afford an explanation for the source of the digestive enzymes necessary to dissolve the flesh of crustaceans before the formation of the pancreas, which is extensively developed in the adult.

## SUMMARY

1. The carp has become adapted to live in either sewage-polluted water or relatively pure lake water.

2. Before adequate control measures can be recommended, it is desirable that the factors influencing the life history be studied.

3. Conditions of spawning are variable and the time in part controlled by the temperature of the water and in part by the time of the year when the eggs mature.

4. The eggs hatch in from four to seven days in the usual warm water, but the period is lengthened when the jars are kept in the shade.

5. The larval life falls into the inactive period, which lasts until the yolk is about absorbed, and the active, when feeding begins. The young fry stage begins when the golden tints are present and the young fish has taken the form of the adult, which occurs from three to four weeks after hatching.

6. The intestine increases in diameter and length through the rapid multiplication of nests of nuclei by the mitotic process in specific regions. The syncytial condition exists with the cells in a normal tissue formation on each surface of the nest which may extend for  $30\mu$  in length.

7. The beginning of the formation of the mucous cells is found in nuclei close to the basement membrane around which cytoplasm collects that gradually protrudes to the lumen of the intestine. In these cells mitochondria are present and secretion masses.

8. Distinct masses, similar to the nuclei, are present in both the mucosa cells and the syncytium which are interpreted as secretion bodies.

LITERATURE ON THE STRUCTURE OF THE DIGESTIVE TRACT  
OF FISHES

For a review of the literature on the structure of the digestive tract of fishes, see:

- BLAKE, I. H. 1930 Studies on the comparative histology of the digestive tube of certain teleost fishes. I. A predaceous fish, the sea bass (*Centropristes striatus*). Jour. Morph. and Physiol., vol. 50.
- DAWES, B. 1930 The histology of the alimentary tract of the plaice (*Pleuronectes platessa*). Quart. Jour. Micr. Sci., vol. 73.
- OPPEL, A. 1897 Lehrbuch der vergleichenden mikroskopischen Anatomie der Wirbelthiere. Schlund und Darm.



# THE INTRANUCLEAR DEUTOPLASM<sup>1</sup> AND THE ORIGIN OF GAMETES IN THE TURBELLARIAN PRORHYNCHUS APPLANATUS KENNEL

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ONE PLATE (THIRTEEN FIGURES)

## AUTHOR'S ABSTRACT

It is shown that in *Prorhynchus applanatus* there exists a type of yolk elaboration which has hitherto not been reported. Formation is within the nucleus through the growth and fusion of nucleoli, but the yolk globule becomes larger than the original nucleolus. The developing individual utilizes the food material contained in the yolk cells in the following order: 1) cytoplasm of the yolk cell, 2) yolk bodies contained in this cytoplasm, 3) intranuclear yolk, 4) nucleoplasm. It is also shown that the germ cells in this form arise from the endoderm by diapypesis, but that the yolk cells are mesenchymal in origin. The sequence of formation of male and female gametes precludes the possibility of the presence of sex hormones such as are found in higher forms.

## INTRODUCTION

Two general types of female reproductive systems are found in lower Turbellaria. These are: a) those without vitellaria and, b) those with vitellaria. As an example of the first type *Stenostomum* might be cited. In this genus the gamete elaborates its own deutoplasm. This deutoplasm is deposited within the cytoplasm of the egg cell which eventually becomes so greatly crowded with yolk granules that the nucleus is not readily seen as it lies among them. In contrast to this gamete, the mature ovum of *Prorhynchus applanatus* is at all times free of deutoplasm. Of those forms with vitellaria, the genus *Prorhynchus* presents perhaps the simplest condition. Here, according to von Graff ('08) and Steinböck ('27), the ovary is divided into several chambers or follicles. Each follicle consists of a germ cell, the ovum,

<sup>1</sup>The terms deutoplasm and yolk are used in this paper, as they seem to be generally used by turbellarian workers, to denote material that is used as food material by the developing organism and which, with ordinary stains and technique, reacts like yolk. It should be understood that no specific chemical tests for yolk have been employed in this work.

surrounded by a group of accessory or yolk cells. It is the latter that form the yolk and hence may be considered a vitellarium. In *Dalyellia* we find a much more complex condition, in that the germarium and vitellarium are not only differentiated, but also spatially separated, so that distinct yolk glands are present. Thus it is evident that the *Turbellaria* exhibit a wide range of variation so far as the female reproductive systems are concerned, varying from forms without vitellaria to those with true yolk glands.

*Prorhynchus*, therefore, so far as its female reproductive system is concerned, occupies an intermediate position among the simpler *Turbellaria*. It was this fact which prompted a study of its ontogeny with the expectation that something might be learned therefrom concerning the origin of the deutoplasm and of the gametes.

#### MATERIALS AND METHODS

*Prorhynchus applanatus* is found in abundance in the streams and ponds about the University. Frequent collections were made and a number of cultures maintained over long periods of time. These animals are easily reared under laboratory conditions. For culture purposes several specimens were placed in a small dish in tap or spring water with a little sediment containing diatoms, desmids, and other small algae. These algae together with various ciliates, especially paramoecia, and small crustacea, which were added from time to time, afforded sufficient food for the animals. Many such cultures may be maintained at the same time, as they require very little attention.

In order to obtain an ontogenetic series, all cultures were examined daily for eggs. These, when found, were removed from the culture and placed in a small dish. They were then examined hourly throughout the day. Newly laid eggs are very opaque, due to the presence of a large number of yolk cells. With the development of the embryo, however, the yolk cells gradually disappear, so that shortly before the emergence of the young individual the egg becomes relatively

translucent, and the young individual may be observed within the capsule. Egg capsules in this state were isolated in depression slides and examined at fifteen-minute intervals so as to obtain the exact time of emergence of the new individual. A series was then made consisting of animals fixed at the following periods: 1) at hatching, 2) three hours after hatching, 3) seven hours after hatching, 4) twelve hours, 5) twenty-four hours, and every twelve hours thereafter, concluding with a nine-day-old specimen. Two or more specimens were fixed at almost all of the indicated periods, so as to provide a check on the work. This proved of value in that variations in the degree of development became manifest after five or six days. These variations became so great that after the nine-day period, the twelve-hour interval as a basis for the series was abandoned, and the remaining stages were taken on the basis of body size and ovarian development.

Specimens were fixed in Zenker's fluid or in Bouin's fluid for from fifteen to thirty minutes each. Zenker's yielded the better results and hence was used in most instances. Sections, made for the most part transversely at  $7\ \mu$ , were stained in Heidenhain's iron haematoxylin (four to twenty-four hours) and slightly counterstained with eosin in 70 per cent alcohol.

#### THE INTRANUCLEAR YOLK

Ludford ('21), working on the mollusc *Patella*, suggests that certain nucleolar extrusions which he finds in the cytoplasm of the egg play a part in yolk formation. Gresson ('29) and others, working on various arthropods, state that in some forms at least nucleolar extrusions passing through the nuclear membrane into the cytoplasm of the egg give rise directly to the albuminous yolk. These are the only instances recorded in available literature of yolk having a nucleolar origin, and in these the nucleolar extrusions which give rise to the yolk occur in the egg cell proper.

Von Graff ('08) describes two chief methods of yolk formation in the Turbellaria. In *Stenostomum*, for instance, the



yolk is formed within the cytoplasm of the egg cell. In *Monoophorum striatum* the immature yolk cell is finely granular. Through the growth and fusion of these small granules much larger bodies which are contained in vacuoles are formed. These constitute the yolk granules in the mature yolk cells. Von Hofsten ('12) points out the fact that in some forms the protoplasm of the yolk cell may serve as food material for the embryo without previously having been organized into definite yolk bodies.

Steinböck ('27) describes the immature yolk cell of *Prorhynchus balticus* as having finely granular cytoplasm. As the cell matures it grows rapidly in size and vacuoles appear in its cytoplasm. The yolk granules are eventually formed within these vacuoles. As the cell increases in size the nucleus also becomes larger. The nucleus contains a nucleolus which in a mature yolk cell normally occupies not more than one-half of the area within the nuclear membrane. Yolk formation in *Prorhynchus applanatus* is essentially the same as in *P. balticus*, according to Steinböck, with this exception, that in *P. applanatus* (as well as in *P. sphyrocephalus* and *P. metameroideus*) the nucleolus increases tremendously in size, until in a mature yolk cell it occupies the entire space within the nuclear membrane and in fact exceeds the boundaries of the original nucleus. These nucleoli Steinböck calls 'Riesennukleolen,' or giant nucleoli. As to the function of the 'Riesennukleolen,' he says:

Vejdovsky (1895, S. 153) glaubt, man habe ihnen weiter keine Funktion zuzuschreiben und meint, "dass man es hier mit einer Hypertrophie der normalen Kernkörperchen zu tun hat, welche schliesslich zur Degeneration der Kerne führt." Für diese Ansicht würde vielleicht die oben angeführte Tatsache sprechen, dass man bei *P. balticus* vereinzelt ähnliche Bildungen antrifft, die bei *P. sphyrocephalus* (sowie *applanatus* und *metameroideus*) zur Regel wurden.

He makes no mention of any possible deutoplasmic function of these 'Riesennukleolen.'

My observations as to the formation of the 'Riesennukleolen' are not entirely in accord with those of Steinböck. In

a very young yolk cell (figs. 1 and 2) there are no nucleoli present in the nucleus—a fact which he recorded. As the yolk cells and their nuclei grow, however, nucleoli appear, and as a rule there are several present in a single nucleus (figs. 3 and 4). These become larger as the cell matures and eventually fuse into a single mass (fig. 5). This mass continues to increase in size until it exceeds the boundaries of the original nucleus (fig. 6). Throughout the whole process the nucleolus stains black with iron haematoxylin, thus reacting like a karyosome. When it has attained full size, however, it is no longer stained by the haematoxylin, but becomes red from the eosin which was used as a counterstain. Its reaction toward stains is now that of a plasmosome. That this so-called nucleolus is a definite deutoplasmic body is shown by a study of its fate in the individual just after emergence from the egg.

At the time of egg laying a membrane is formed enclosing the entire follicle, which then passes out through the genital pore. A newly laid egg, then, consists of a germ cell surrounded by a number of yolk cells and the whole enclosed in a membrane. Due to the fact that the egg membrane is extremely impervious to fixatives, no study was made on development within the egg. The opaque nature of the capsule, due to the light-refracting yolk particles present therein, for the most part precluded the possibility of observation on the earliest stages of development also. The yolk gradually disappears in the later stages, however, and it is possible to see something of what takes place within the membrane. Some years ago, H. E. Hayden, Jr., of this laboratory, while studying a late egg capsule of *P. applanatus*, observed that the young specimen within the capsule was engaged in the active ingestion of yolk material, a good deal of which was still present at this stage. This ingestion was accomplished by means of the prehensile pharynx which was protruded through the oral aperture in such a manner as to take in the yolk particles. This observation has since been made by me also. That we are not here dealing with an embryonic pharynx which is developed solely for the purpose of yolk

ingestion and is later replaced by the true pharynx, as Curtis ('02) and other workers describe in *Planaria*, is evident from the fact that animals slightly older than those seen ingesting yolk (which were forced out of the egg capsule before hatching) show the same pharyngeal structure in section as the adult. Nor is there a trace of any other pharyngeal structure.

The part played by the 'Riesennukleolen' of the yolk cells, which constitute the intranuclear deutoplasm, in the development of the young individual was determined by a study of the ontogenetic series. These deutoplasmic bodies were found in the enteric lumen in specimens which were forced out of the egg capsule previous to their normal emergence. In these very young specimens they have been only slightly attacked by digestive juices and are just beginning to stain with haematoxylin, so that they have a reddish black appearance. In animals fixed at hatching they stain black and this staining reaction continues until their complete disintegration. In the youngest animals obtainable the cytoplasm of the yolk cell which surrounded the deutoplasmic bodies or yolk crystals has already been digested away from them. Specimens fixed about twelve hours after emergence from the egg proved to be best for studying the disintegration of the yolk crystals, as in these almost all desired stages could be found. Figures 7, 8, 9, and 10 show successive phases in this disintegration. Throughout the series the crystal is surrounded by a granular plasma. This seems to be nucleoplasm, showing that the hypertrophy of the normal nuclear bodies does not lead to degeneration of the nucleus, as suggested by Vejdovsky ('95) and Steinböck ('27). Thus the food stored in the egg capsule with the germ cell is utilized in the following order: 1) the cytoplasm of the yolk cells, 2) the yolk granules contained in the cytoplasm, 3) the intranuclear yolk, and, 4) the nucleoplasm.

#### THE ORIGIN OF GAMETES

The question of the origin of gametes is one which most of the recent workers on the *Turbellaria* seem to have avoided.

In view of the contrasting opinions of earlier workers on this subject, it is rather surprising that it should not have received more attention in recent years. Lang ('84) was apparently one of the first to advance the theory of the endodermal origin of gametes in turbellarian forms. He found that in the polyclads and in the triclad *Gunda segmentata*, the egg cells arose from the endodermal epithelium. This view was supported by the work of Korschelt ('84) on *Dinophilus apatris*, and that of Böhmig ('90) on *Plagiostoma dioicum* and *Pl. bimaculatum*. Further, Fuhrmann ('94) says of *Stenostomum leucops* that "Die Ovarien entsprossen dem Darmepithel und sind deshalb von der Muscularis des Darmes umgeben." On the other hand, Keller ('94), working with another species of *Stenostomum*, *S. langi*, says:

Das Ovarium entsteht medio-ventral im Pseudocöl, etwas vor der Körpermitte. Die Stammzellen bilden ein ovales Zellenpolster, welches durch Resorption der Zellmembran in das Stadium eines sog. Syncitiums übergeht. Am vorderen und hinteren Ende dieses Plasmakörpers beginnt sodann die Abspaltung der Eier.

Duplessis ('84), who, like Böhmig ('90), worked on *Plagiostoma*, states that in *Pl. lemani* the male and female gametes, as well as the yolk cells, probably arise from the mesenchyme. Thus in two instances conflicting opinions as to the origin of gametes were held by men working with different species of the same genus.

Among those who have described the endodermal origin of gametes in the vertebrates, Swift ('14), Allen ('06), Woods ('02), and Vanneman ('17) might be mentioned. Woods ('02), in *Acanthias*, and Vanneman ('17), in the armadillo *Tatusia novemcincta*, trace the migration of the primitive germ cells through the endodermal wall of the embryo to their ultimate location. The passage through the endodermal epithelium is accomplished by means of amoeboid movements on the part of the germ cell itself—a process known as diapedesis. There has been a great deal of work done on the origin of gametes in vertebrates in recent years, but apparently none has been done along this line in the Turbellaria

since 1894. Thus Steinböck ('27), in his "Monographie der Prorhynchidae," does not say which of the three primitive tissues gives rise to the germ cells in these forms. In view of this fact, the following observations are recorded.

The ovary in *Prorhynchus applanatus* is located ventral to the diverticulated enteron and posterior to the pharynx in the median portion of the body. It is follicular in construction, consisting of seven to twelve follicles, each of which contains a single germ cell surrounded by a number of accessory or yolk cells. The anterior follicle is differentiated before the young individual emerges from the egg capsule (fig. 1). The testes, as described by me in a previous paper ('29), are ventral and lateral to the enteron, and extend from a region near the posterior end of the pharynx backward to about the middle of the body or beyond. They consist of more or less paired follicles, usually six or seven in number, each one of which contains many spermatogonia and produces numerous spermatozoa. The testes, together with the other parts of the male reproductive system, usually develop after the female system has attained maturity. Occasionally, however, the male system will develop and reach maturity while the female system is still so immature that it can scarcely be seen in a living animal. Thus this species is essentially protogynous, but protandrous individuals may appear. Further, the organs of either sex may develop independently, without regard to those of the other, so that the presence or absence of one gonad neither determines nor inhibits the development of the other. This seems to preclude the possibility of sex hormones such as are found in the higher forms. Sexually mature individuals of this species may be found at any time of the year, but female specimens are always much more common than male. From this fact it appears that the ovary is persistent, while the male organs are transitory.

The testes and ovary contain the germ cells which, after a series of maturation divisions, produce the spermatozoa and ova. These germ cells arise from the endoderm by means of diapedesis. In animals fixed about twelve days after emer-

gence from the egg, shortly before the appearance of the male reproductive system, many stages of diapidesis may be observed. An endodermal cell begins first to send small pseudopods through the basal membrane of the enteron. Gradually, by means of amoeboid movement, the entire cell leaves the endoderm and makes its way into the adjacent mesenchyme. At times only one, but frequently two (fig. 12) or three cells will be migrating at a given point. When they are free in the mesenchyme, these cells collect into follicles, and in this manner the testes are formed. Diapidesis occurs for the most part from the lateral and ventrolateral portions of the endodermal epithelium, as would be expected, since it is these regions which are in closest proximity to the testes of the adult. A few cases of dorsal and ventral diapidesis were observed. Cells migrating from the dorsal portion of the endodermal epithelium upon reaching the mesenchyme probably continue their amoeboid motion after a change of direction, so that they finally find their way into the lateral mesenchyme. Here they join with the cells from the lateral region of the endoderm in the formation of a testis. Ventral diapidesis, on the other hand, gives rise to the oogonia. Since there are always relatively few oogonia, it is to be expected that there should be few cases of ventral diapidesis. Further, none of these were found in regions which showed a definitely differentiated oogonium. Some time before it actually sends out pseudopods, the endodermal cell (fig. 11) has begun to undergo changes preparatory to becoming a germ cell. The nucleus enlarges and becomes more coarsely granular, while the nucleolus disappears. As the cell migrates (fig. 12, *D.C.*) these changes become more and more pronounced until it soon becomes a typical spermatogonium (fig. 13).

The yolk cells of the ovary, on the other hand, seem to be mesenchymal rather than endodermal in origin. In several instances I have seen mesenchymal cells near, but entirely outside of the ovary, which had well-developed intranuclear yolk within them. On the other hand, no endodermal cell has

been observed to contain intranuclear yolk. Further, no ventral diapedesis has been observed in regions which showed a distinctly differentiated oogonium, regardless of whether the ovary in that region was mature or not. The single ovarian follicle (fig. 1), found in an animal shortly after emergence from the egg capsule, shows the germ cell with several ventral accessory cells. No accessory cells are present dorsal to the germ cell as yet. It seems, then, that an endodermal cell migrating in a ventral direction leaves the endoderm and, pushing the mesenchymal cells downward, takes its place between them and the enteron. These mesenchymal cells then become the earliest accessory cells. Later, other mesenchymal cells come to lie between the germ cell and the enteron. They, too, become modified into yolk cells, so that in time a mature ovarian follicle is formed.

#### DISCUSSION

Variation is a matter of fundamental interest in biology. The wide variation shown by nidamental cells such as yolk cells in the Turbellaria is therefore noteworthy. It has already been pointed out that some Turbellaria possess no yolk cells, so that in these forms the deutoplasmic material is produced within the egg cell. In *Prorhynchus* the accessory cells of the ovary produce the yolk, but they are not morphologically discrete from the germarium. On the other hand, distinct yolk glands are present in *Dalyellia*. Yolk cells, which are mesodermal in origin, constitute only a single instance of the marked variation which occurs in all mesodermal derivatives of Turbellaria. Thus one who had worked exclusively with members of one order of Turbellaria would probably encounter great difficulty in identifying the mesodermal derivatives in representatives of another order. This is in striking contrast to the condition that obtains in vertebrates, where relatively little variation is found. Even in two groups as widely separated as the fish and the mammals, the mesodermal derivatives are quite similar.

Biologists have sought to explain variation and its causes along two distinct lines. Some maintain that those of a somatic nature arise as a result of a changeable or unsuitable environment, while others attribute them to germinal variations and to the building of new germinal complexes. In the Turbellaria, as already noted, the mesenchyme and its derivatives show a wide range of variation. This tissue is well insulated from the exterior and hence has little or no contact with a highly variable environment. It seems illogical to suppose that a tissue which is so well protected could show so much variation solely as a result of external influences. Hence, the condition of the mesenchyme in the Turbellaria offers strong evidence in support of the contention that somatic variations arise as a result of germinal variations or through the building of new germinal complexes.

#### SUMMARY

It has been shown that in *Prorhynchus applanatus* there exists a type of yolk elaboration which has hitherto not been reported. Formation is within the nucleus through the growth and fusion of nucleoli; but the yolk globule becomes larger than the original nucleus. The developing individual utilizes the food material contained in the yolk cells in the following order: 1) cytoplasm of yolk cell, 2) yolk bodies contained in this cytoplasm, 3) intranuclear yolk, and, 4) nucleoplasm. It has been shown also that the germ cells in this form arise from the endoderm by diapedesis, but that the yolk cells are mesenchymal in origin. The sequence of formation of male and female gametes precludes the possibility of the presence of sex hormones such as are found in higher forms.

I am very grateful for the valuable assistance rendered me by Dr. William A. Kepner, under whose direction this research has been carried out. I also wish to express my appreciation for his suggestions to Dr. H. E. Jordan, of the Department of Embryology.



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## PLATE 1

### EXPLANATION OF FIGURES

1 Cross-section of ovarian follicle in animal twelve hours after emergence from egg capsule.  $\times 1000$ . *N*, germ-cell nucleus; *NA*, nucleus of accessory cells.

2 Oldest accessory cell of ovary which contains no nucleolus.  $\times 1000$ .

3 to 5 The growth and fusion of nucleoli to form intranuclear yolk. *D*, deutoplasmic granules of cytoplasm; *Nc*, nucleoli.  $\times 1000$ .

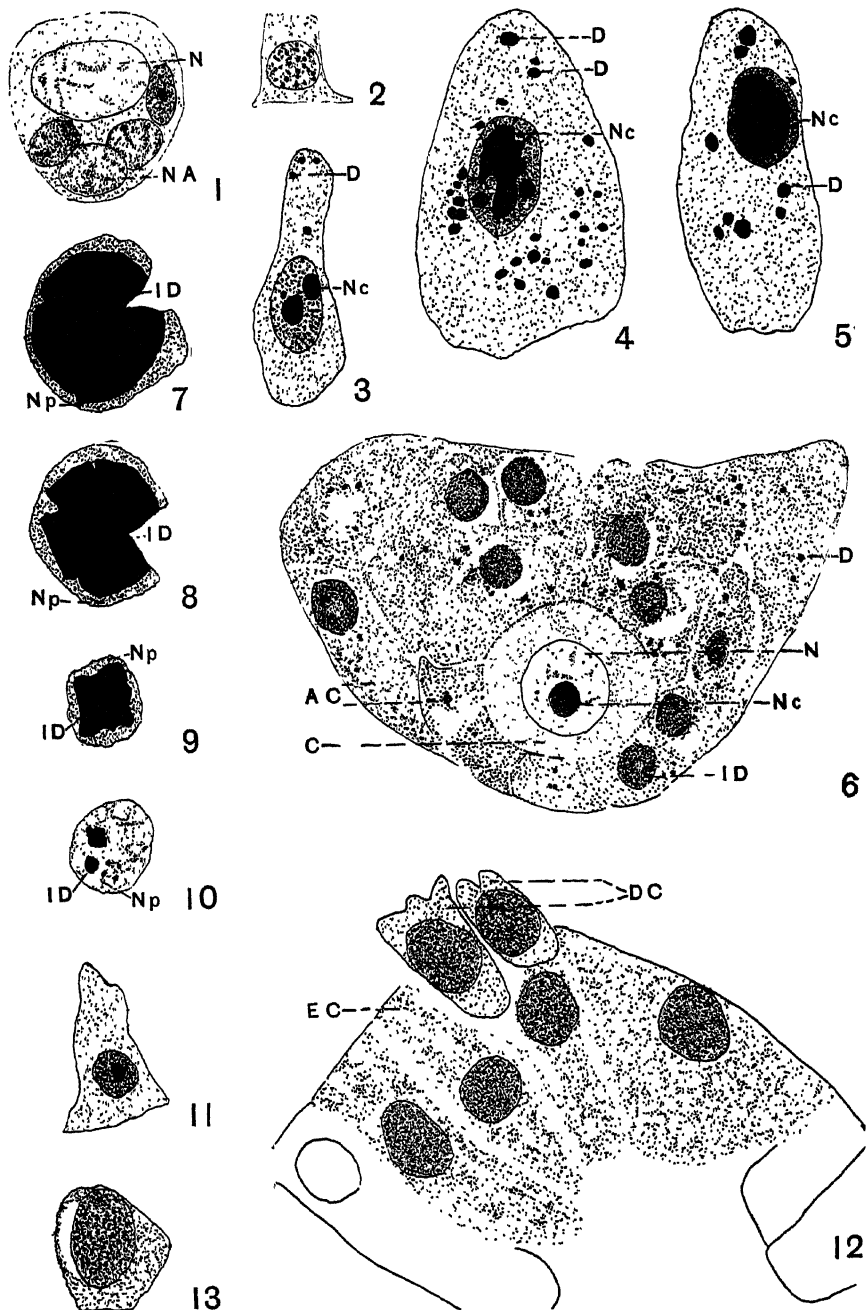
6 Cross-section of mature ovarian follicle. *AC*, accessory cells; *C*, cytoplasm of ovary; *D*, deutoplasmic granules within cytoplasm; *ID*, intranuclear deutoplasm; *N*, nucleus of germ cell; *Nc*, nucleolus of germ cell.  $\times 375$ .

7 to 10 Stages in the disintegration of intranuclear deutoplasm within the enteric lumen of a young specimen. *ID*, intranuclear deutoplasm; *Np*, nucleoplasm.  $\times 1000$ .

11 Typical endodermal cell of a region not showing diapedesis.  $\times 1000$ .

12 A portion of the endodermal epithelium showing diapedesis. *EC*, endodermal cells; *DC*, diapedetic cells.  $\times 1000$ .

13 A typical early spermatogonium.  $\times 1000$ .





# A DETAILED STUDY OF THE ENDODERM OF HYDRA

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SIX TEXT FIGURES AND SIX PLATES (EIGHTEEN FIGURES)

## AUTHOR'S ABSTRACT

The flagella of the endodermal cells of hydra have their origin in the form of a cytoplasmic spherule which appears on the cell membrane just above the blepharoplast. This spherule moves outward, and in doing so forms a small cylinder, which, in time, flattens down into a ribbon-like flagellum, the edge of which is slightly thickened. Endodermal cells were found to bear from one to five of these flagella. Dissociated endodermal cells become amoeboid and are able to move about quite freely, taking up particles of food with pseudopods; these cells are also able to elaborate flagella. In the normal well-fed hydra the endodermal cells are found to fragment endogenously; these endogenous fragments pass to the tentacles and other outlying regions, where they are taken up by the endo-epithelial cells lining these regions. The endoderm is frequently thrown into villi of quite large size: these villi, when studied over a period of days, are found to deteriorate; the cells which composed them wander to the tentacles, buds, and basal regions by means of their flagella and amoeboid movement, where they deteriorate, the cell particles being taken up by the epithelial cells lining these areas.

The endoderm extends as a lining throughout the various regions of a hydra's body. The three anatomical regions of this polyp are: 1) the oral end bearing the mouth, peristome, and tentacles; 2) the middle region; 3) the basal region. Each of these regions has its peculiar endoderm. In their work upon the oral region, Kepner and Hopkins ('24) described in detail the peristomal endodermal gland cells that lie about the mouth. They also demonstrated in this work the presence of sphincters at the bases of the tentacles, these sphincters operating against pressure from the enteron toward the tentacle, but not in the opposite direction. Hadži ('09) studied the middle region intensively and found that this region of the endoderm presented three types of cells: 1) the epitheliomuscular cells which were capable of ingesting small food particles; 2) gland cells, which lay down enzymes upon larger masses of food within the enteron; 3) sensory cells. Hadži, in this respect, followed Schneider ('90 and '02) very closely; the latter having seen that each of these types of cells, except the peristomal ones, bore one

or two flagella. Burch ('28) carried this one step further, and demonstrated that the flagella of the three types of endodermal cells arise from blepharoplasts. Within the basal region of the polyp the endoderm is highly vacuolated in a living specimen, giving the basal third of the body a less opaque appearance. Kepner and Miller ('28) described a new histological region of the endoderm lying in the fundus of the enteron. These cells are not highly vacuolated, as are the lateral cells of the base, and resemble the epithelium of the middle endodermal region. Gland cells were not found to be associated with them, neither were they described as bearing flagella. There are thus found in this polyp's endoderm peristomal gland cells, vacuolated cells within the lateral wall of the basal third, and epitheliomuscular cells containing food vacuoles within the fundus of the enteron and lining the oral two-thirds of the enteron.

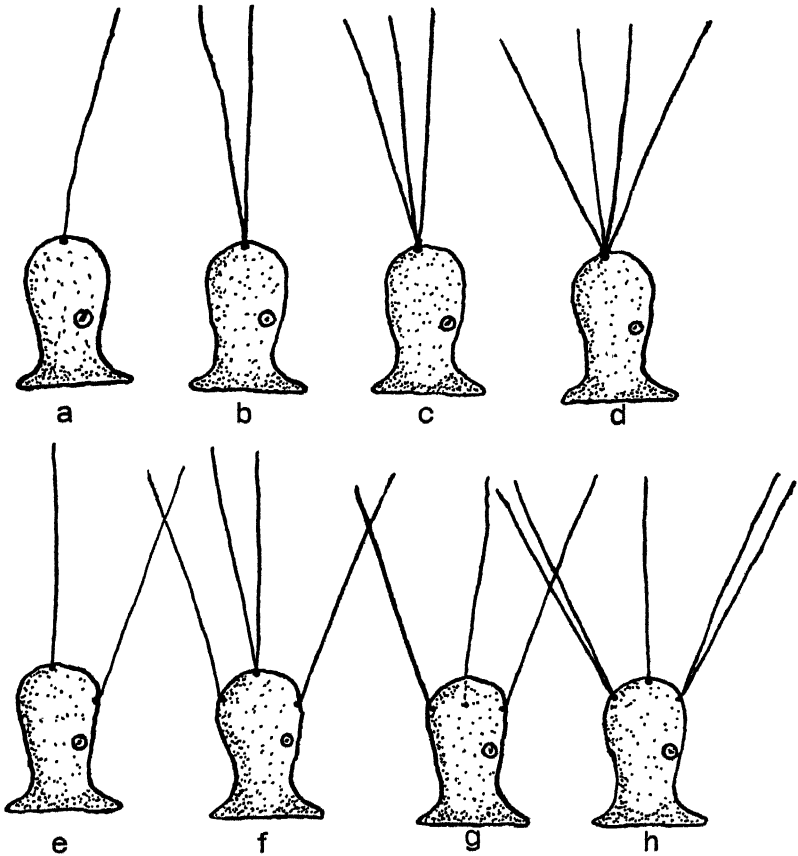
However, when one studies macerated material such wide distinctions cannot be made. For example, the epitheliomuscular cells, which are torn loose from the mesoglea of the oral two-thirds and from that of the fundus of the enteron, upon maceration, cannot be distinguished from each other. Macerations show only a distinct differentiation of peristomal gland cells, sensory cells, and secretory cells of lateral endoderm. But even these cells are not as sharply defined in maceration preparations as in sectioned and stained specimens. My study of the endoderm was confined, first, to the flagella and, secondly, to the independent and mutual activity of endodermal cells.

#### OBSERVATIONS UPON FLAGELLA

The intimate study of the endodermal cells led to some interesting observations concerning the distribution, structure, functioning, and origin of their flagella.

Schneider ('02) inferred that flagella were generally distributed except for the peristomal gland cells. I have found that even these cells bear flagella. Previous observers have indicated that each cell may bear one or two flagella. Cells

were seen in this study that presented as many as five flagella. These flagella, of course, were borne only upon the exposed distal ends of the cells. They were, however, grouped in various manners upon their respective cells. Some cells



Text fig. 1 Diagram to show various methods of insertion of flagella of the endodermal cells of hydra.

have as many as four flagella arising from a single blepharoplast (text fig. 1, *d*) or as many as five assigned to three blepharoplasts, as in text figure 1, *h*. The position of the blepharoplasts within the cells also varies. The blepharoplast is always found immediately beneath the cell membrane, but the blepharoplast is not always terminal. It may be



subterminal and lateral to cells that protrude from the general surface of the epithelium (text fig. 1, *e*, *f*, *g*, and *h*).

The shape of the flagellum of hydra is unusual. Ordinarily flagella are described and depicted as being either cylindrical or conical protoplasmic processes. Hydra's flagellum is a flattened structure. It is ribbon-like; the margin of the ribbon bears a selvage-like thickening (pl. 1, fig. A). The flagella of the tentacular endodermal cells are much shorter than those of the endodermal cells from the body proper. The tentacular flagella measure an average of  $30\ \mu$  in length and  $1.9\ \mu$  in width, while those of the endodermal cells from the body proper average  $65\ \mu$  in length and  $1.2\ \mu$  in width.

The undulating ribbon-like flagellum of hydra does not arise as a short flattened or ribbon-like process. I have not seen the origin of a blepharoplast, but the flagellum always has its inception in relation to a blepharoplast. The first step in flagellum formation is the growth of a spheroidal bud of clear, homogeneous cytoplasm that projects from the cell membrane above the blepharoplast (pl. 1, fig. B). This cytoplasmic spherule begins moving outward from the surface of the cell and, in doing so, forms a small cylindrical tube of clear cytoplasmic material, which gradually flattens down into a ribbon-like contour (pl. 1, fig. C). As the cytoplasmic spherule moves outward, it becomes smaller and smaller, the material being apparently used up in the elaboration of the new flagellum. Eventually the cytoplasmic spherule is entirely spent, and the new flagellum is complete. Before this process has run its course, a slow rhythmical movement is displayed by the flagellum. Very often two or more of these flagellary rudiments have been observed arising from the same blepharoplast (pl. 1, fig. D). In fact, upon several occasions, these cytoplasmic spherules have been observed arising from blepharoplasts in which were inserted two or three already active, complete flagella. In dissociated material, the flagella have been seen to arise, to be completely formed, and to begin their actions in periods of from one

hour to an hour and a half (pl. 1, fig. E); while, on uninjured endodermal surfaces of a complete polyp, they have been seen to be completely formed and to become active in from fifteen to thirty minutes. The actual time intervals that were involved in the observation of a developing flagellum are given in plate 2.

These flattened flagella strike the water with their faces and not with their edges. They do not, therefore, inscribe by their action a conical area, but they beat the water by a series of undulations (pl. 1, fig. F). This action sets up two lateral vortices which converge at the base of the flagellum and then are carried distally along the undulating flagellum (pl. 1, fig. F). At first sight this would suggest that food particles would be carried from the cell tip, but what actually happens is that the particles are brought into contact with the subterminal margin of the cell. This region is perhaps the most amoeboid part of the cell. When a food particle comes into contact with this portion of the cell body, it is engulfed by pseudopodia. The direction of the vortices maintained by the flagellum is such that one can infer that oxygen may also be carried to the cell tips and metabolic wastes carried from them. When flagella are grouped or when there are more than one upon a cell, each flagellum maintains its own vortices. In other words, the flagella do not act in unison, and each dominates its own respective area.

#### AMOEBOID MOVEMENT OF THE ENDODERMAL CELLS OF HYDRA

Through pseudopodial processes the endodermal cells take up more or less solid nutritive particles after the manner of Rhizopoda, as is to be seen easily in transparent specimens. The endodermal cells, which are chiefly, if not entirely, responsible for the work of digestion and absorption, are amoeboid in character, throwing out larger or smaller pseudopods from their free borders as occasion may demand. At times the endodermal cells appear to fuse together so as to form something of the nature of a syncytium, a mass of protoplasm with several nuclei, but lacking in dividing mem-

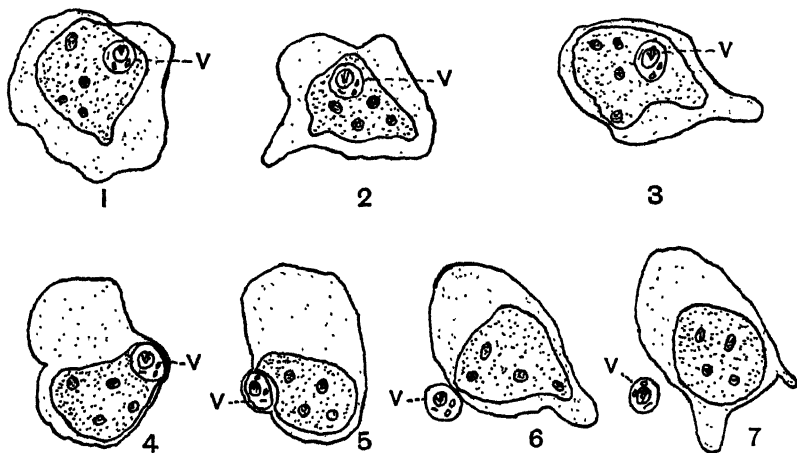
branes. This formation of syncytia may be seen in both living and fixed endoderms. As a rule, the particles taken into the protoplasm of the cells are of small size, easily handled by the pseudopodia of the cells. The pseudopods concerned with the process are formed on the ends of the cells which are exposed to the gastrovascular or coelenteric cavity. Consequently, the endoderm of hydra must be listed in the category of amoeboid epithelia. This observation having been made, it was decided to make a study of these cells after they were dissociated. By careful maceration, endodermal cells were separated from the supporting mesoglea. Amoeboid movement was frequently supplemented by flagellary movement. Indeed, the first actions after separation consisted of the free swimming of these cells, if they were equipped with flagella and not greatly pressed between the slide and the cover-glass. This ability to swim about was very marked and under certain conditions lasted for quite a while. Upon coming in contact with other cells, large pieces of ectodermal or endodermal tissue, or areas where the cover-glass and slide were very close together, they were found to be able to become very amoeboid, and in this manner, they extricated themselves from apparently disadvantageous positions. After doing so, they were observed, in many cases, to round off and swim away by means of their flagella.

Very often, as a result of rapid pseudopodial formation, the cells carried their flagella out upon the end of a pseudopod which was too slender to support them, and the flagella were whipped off. The flagella then swam rapidly away, and the cell continued, henceforth, to be amoeboid (pl. 3, figs. 1 and 2; pl. 4; pl. 5, fig. A).

The pseudopodial formations vary greatly in character; some are large and lobose, others long and filamentous. The cytoplasmic contents flow into them, as they arise, with great rapidity. By means of these pseudopods, the free endodermal cells are able to travel quite long distances. The pseudopods of a free cell, which has lost its flagella, usually form most rapidly on the original distal surface, indicating a polarity

referable to the cell's original position in the epithelium; although, after the cell has been amoeboid over a long period of time, this polarity disappears and pseudopods are formed over the entire surface of the cell membrane to such an extent that the cell takes on many strange shapes and forms (pl. 6, figs. 1, 2, and 3; pl. 3, fig. 3).

While amoeboid, these endodermal cells were found to be able to pick up particles of food and to egest apparently indigestible particles, just as they were able to do while associated with each other when in an epithelium (pl. 5, fig. B; pl. 3, figs. 4 and 5; text fig. 2).



Text fig. 2 Camera-lucida drawing of an isolated endodermal cell of green hydra eliminating a vacuole (*v*) in which apparently indigestible particles are present. Drawings made at intervals of five minutes.

Thigmotactic stimuli led freshly dissociated amoeboid endodermal cells to coalesce. This power to coalesce, however, is not long maintained.

Free amoeboid cells and coalescence have thus been observed in material outside the hydra's body. If, however, a piece of the endoderm be torn from the mesoglea and thrown into the coelenteron, the cells of this fragment will become amoeboid. Again, all of the cells involved in resorption display amoeboid movement when they lie free within the coelenteron.

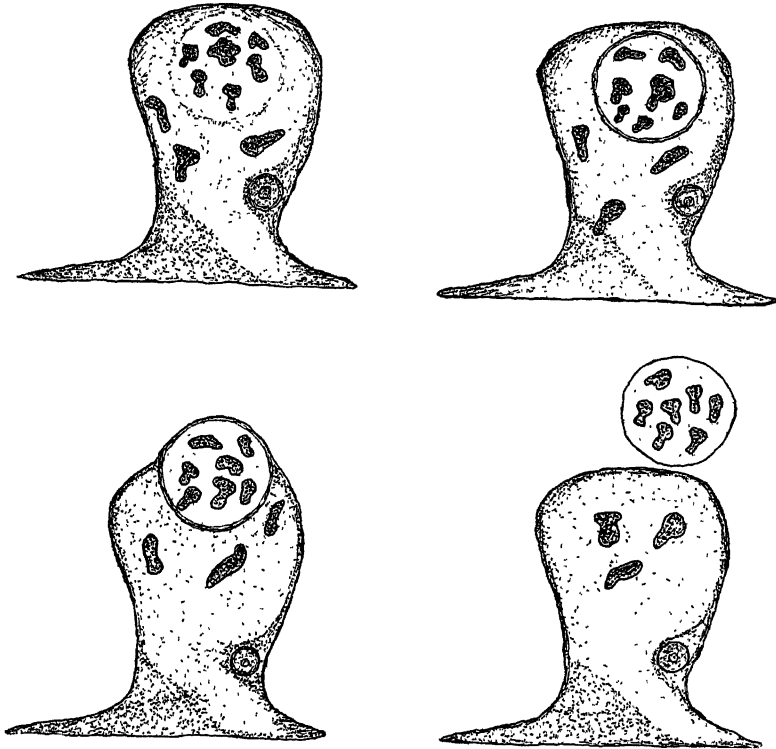
Coalescence, too, may be observed within a complete hydra as well as in isolated tissue elements. For example, a cell of the body proper may leave its place in the endoderm and travel to either a bud or a tentacle and there coalesce with the free ends of an endodermal cell. This fusion, however, is not permanent; for the migrating cell, thus anchored, eventually breaks down.

#### INTERNAL TRANSPORTATION

The migration of free endodermal cells represents one method of internal transportation of material within hydra. Diploblastic anatomy does not lend itself to the development of blood or lymph vessels. The peristalsis of the hydra, it is true, greatly agitates the fluid contents of the coelenteron. Digested material and small fragments of food are thus distributed freely to all endodermal surfaces of the polyp. Small particles of food are taken up by the epitheliomuscular cells to be digested by them within food vacuoles. In time, all the digested food within the coelenteron is absorbed by the endoderm. This tissue, thus, takes up all food for the entire colony of cells. The food absorbed is, in part, assimilated by the endodermal cells and in part handed over to the ectodermal cells by diffusion and osmosis. All this has been generally known, but, in addition to this, two other methods of internal transportation were observed in this study: they are, first, endogenous fragmentation; secondly, the disintegration of villi, which arise from the endodermal lining of the body.

By endogenous fragmentation many of the well-fed epitheliomuscular cells in the endoderm of the upper two-thirds of the body cast out capsule-like fragments which have been formed just beneath their membranes. These capsules contain food bodies. They are observed to be formed under the membranes of cells which exhibit very little pseudopodial action. These cells are not, therefore, active in collecting food from the coelenteron. The fragments about to be enclosed within a capsule are first seen in a group near the

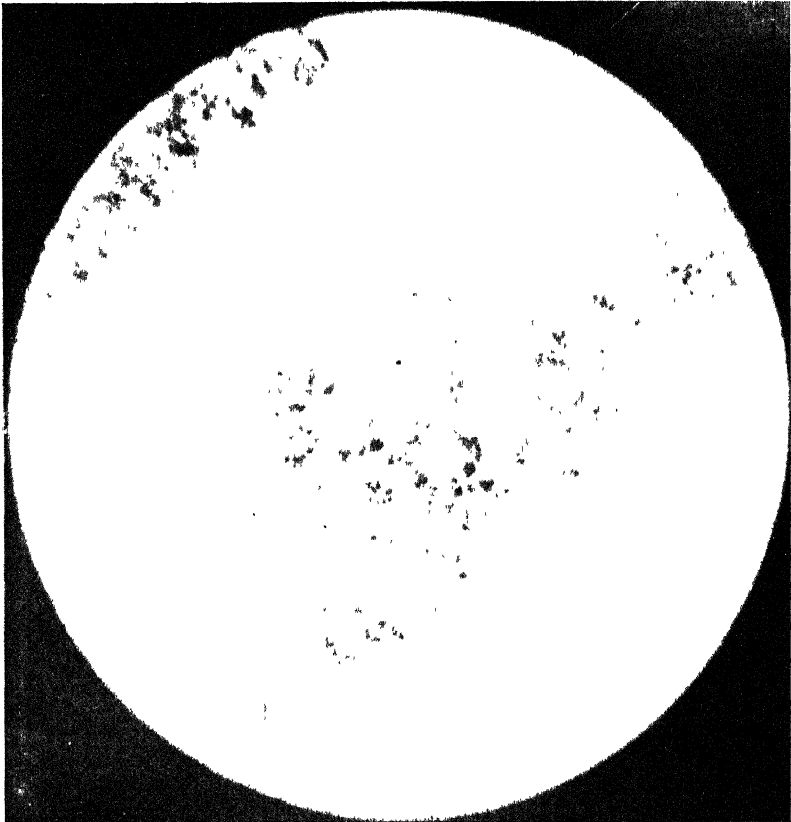
distal end of the cell. About this group there very soon appears a slight membrane which entirely encloses it (text fig. 3). The capsule, thus formed, eventually breaks through the cell membranes and floats, by peristaltic movement of the body of the hydra and the action of the flagella, to various



Text fig. 3 The process of endogenous fragmentation. Drawn from living specimen; time consumed, forty-five minutes.

outlying regions of the coelenteron; here it enlarges slightly, finally bursting, thus allowing the stored food products to escape, which are, in their turn, utilized by the endodermal cells lining these regions. This endogenous fragmentation is best seen and most frequently observed in well-fed hydras which are not engaged in the process of intercellular digestion of some animalcule which has lately been taken in as food.

In the study, both of prepared sections and of living animals, large villi, which have arisen from the endodermal lining of the upper two-thirds of the body, are often encountered (text figs. 4 and 5). These villi are composed of epitheliomuscular cells, secretory cells, and nerve cells. The



Text fig. 4 Villus from the endoderm of brown hydra.

mesogleal core of the villus becomes more and more extensive as the villus ages. The villi are large and lobose, and their surfaces are equipped with many flagella. Just how and why these villi have arisen is rather difficult to determine. It has been suggested that the formation of villi is not normal. Be that as it may, the following observations suggest that

under certain conditions they play an important rôle in the life of the polyp. When the villi are studied in the living animal over a period of days, they are found to disintegrate (text fig. 6). The cells of which they are composed are seen

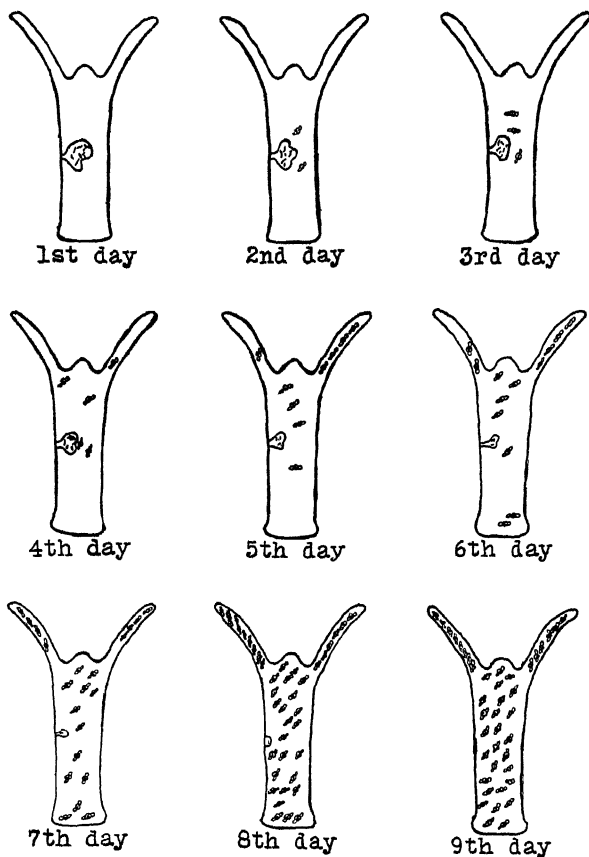


Text fig. 5 A typical villus which has just begun to deteriorate.

to migrate, when free, to various regions of the coelenteric cavity by the action of flagella, both their own and those of the endodermal cells; by amoeboid movement; and by peristaltic activity of the hydra. Upon leaving the villus, the cells were observed to have become very amoeboid, then highly vacuolated, and, eventually, to have burst. The



resultant contents and fragments are picked up by the endodermal cells which lie in the vicinity in which the former cells have broken down. In this manner, the endoderm in the outlying regions has access to food resulting from the disintegration of the villi, and from the migration and breaking down of the cells which composed it.



Text fig. 6 A diagram showing the disintegration of villus during a period of nine days. Note areas to which cells of disintegrated villus have migrated.

## DISCUSSION

From the standpoint of its diploblastic nature, hydra must make cellular adjustments that are peculiar to it. These adjustments are imposed upon diploblastic organization because of the lack of mesodermal tissue out of which to elaborate transportation structures. Transportation methods in hydra, therefore, involve the activity of flagella, independent cells, and aggregates of cells.

By their universal distribution on the endodermal cells and by their concentrated action, it can readily be inferred that the flagella of hydra aid greatly in aeration of the entire endoderm and facilitate the transportation of food particles and alimentary fluids throughout the body proper. The flagella, therefore, are important to the metabolism of the animal, and yet, because of their delicate structure and the conditions under which they exist, they are quite likely to be lost. They are lost under three conditions: 1) through auto-amputation, 2) through accidental abrasion, 3) when the endoderm is temporarily transformed from an epithelium into a syncytium. Correlated with this tendency to lose flagella there is the capacity, on the part of the endodermal cells, of readily reforming flagella to take the place of those lost. It is thus seen that the endoderm of hydra is not a mere secreting and absorbing tissue. The endoderm of hydra is in addition a very important transportation tissue, as is displayed by the functioning of the flagella.

The action of flagella as distributing agents, however, while important, is not wholly adequate, for in specimens which display flagellary activity the endodermal cells of the tentacles and basal region may be highly vacuolated, while the endoderm of the remainder of the body is richly supplied with food in inclusions. Flagellary distribution of food, therefore, is supplemented by the formation and discharge of endogenous fragments from the endo-epithelium of the oral two-thirds of the polyp. These fragments are sent out into the remotest parts of the coelenteron. Thus it seems that hydra's endoderm is still further removed from being a mere

secreting and absorbing tissue. It is in a second manner functioning as a transporting tissue through the elaboration of endogenous fragments. The loss of flagella and their reformation together with the elaboration of endogenous fragments are carried on at the expense of the cytoplasm.

The hydra, then, is an animal composed of two tissues in which the cell colony as a whole is maintained through a high degree of cooperation. This cooperation is carried to the point where cytoplasm of certain of the endodermal cells may be consumed, lost, or destroyed for the welfare of the cell colony.

#### SUMMARY

1. Flagella are universally distributed upon all types of cells through the entire endodermal lining of hydra.

2. These endodermal cells may bear from one to five flagella, these arising from blepharoplasts which may be located distally or subterminally. More than one flagellum may arise from the blepharoplast.

3. The shape of hydra's flagellum is peculiar in that it is ribbon-like, the outer edges bearing selvage-like thickenings.

4. The flagella of the endodermal cells of the body proper are longer, but more slender, than those of the endodermal cells of the tentacles. The measurements of a flagellum from the body proper and one from the tentacle indicate that they may contain approximately the same amount of material.

5. The flagella arise as cytoplasmic spherules on the membrane above the blepharoplast. This spherule moves outward from the surface of the cell; the cytoplasmic cylinder which is formed flattens down into a ribbon-like structure which begins active movement when almost complete.

6. Flagella may be elaborated by dissociated endodermal cells.

7. The action of a flagellum creates vortices which converge laterally upon the distal surface of the cell.

8. The endodermal cells of hydra become amoeboid when removed from the mesoglea.

9. Dissociated endodermal cells of hydra are able to move about and pick up food particles by means of pseudopods.

10. The pseudopodial formations vary greatly in character. Some are large and lobose, others long and filamentous.

11. Endodermal cells are positively thigmotactic and coalesce freely.

12. Internal transportation takes place within the coelenteric cavity of hydra in five ways, namely: by osmosis and diffusion; by peristaltic movement of the body; by flagellary activity; by endogenous fragmentation, and by the disintegration of villi.

#### ACKNOWLEDGMENTS

This work was carried on under the supervision of Dr. Wm. A. Kepner and Dr. B. D. Reynolds, Miller School of Biology, University of Virginia, to whom I am grateful for much valuable aid and criticism.

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## PLATE 1

### EXPLANATION OF FIGURES

A The tip end of a newly elaborated flagellum. Note the selvage-like thickening of the edge.

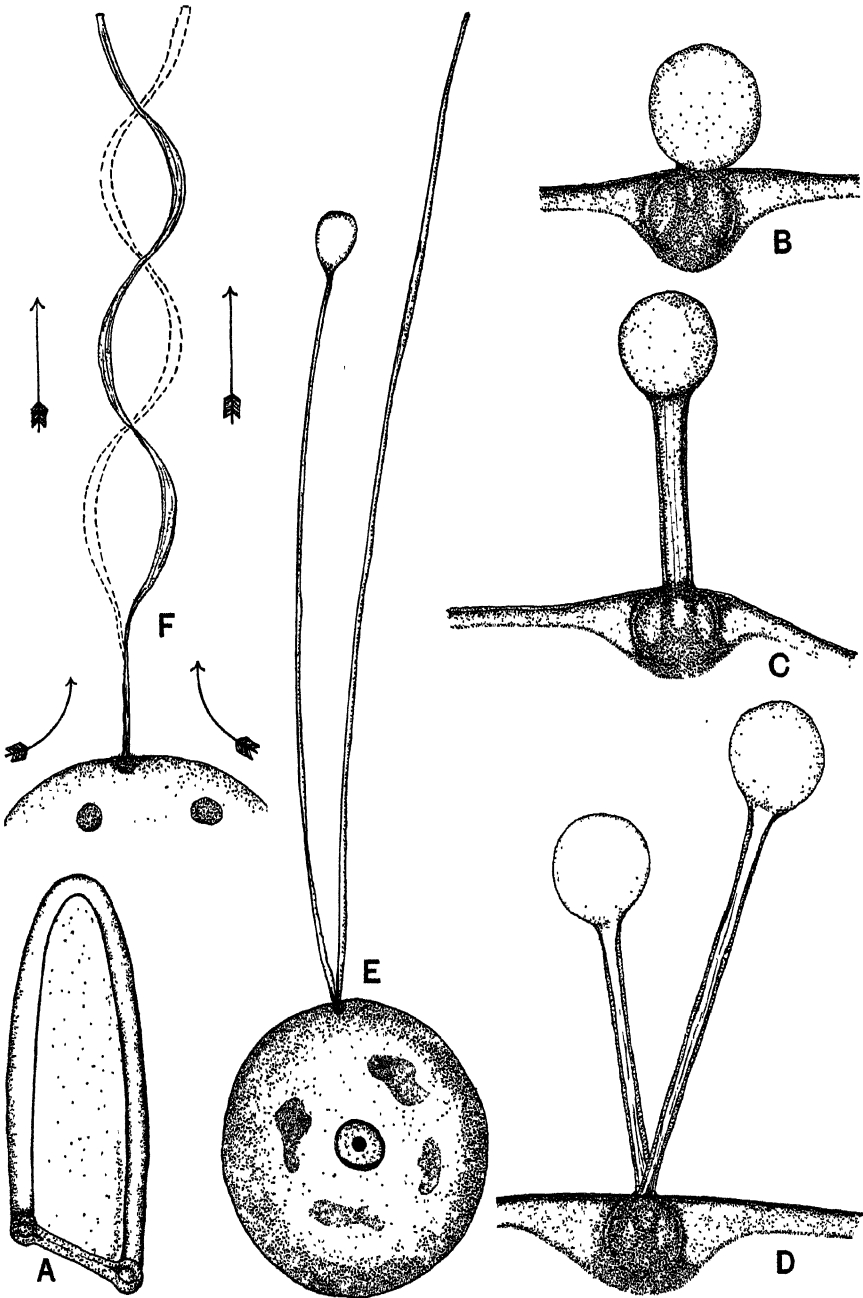
B The first appearance of the cytoplasmic spherule that is to be elaborated into a flagellum. The spherule is on the cell membrane exterior to the blepharoplast.

C The second stage of flagellum formation.

D Two flagella arising from a single blepharoplast which is just under the cell membrane.

E An isolated endodermal cell of brown hydra with one mature flagellum and another in the process of development.

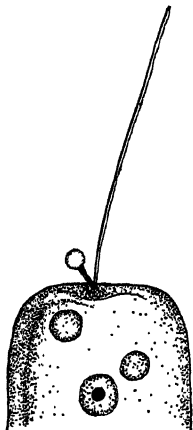
F Diagrammatic sketch of the direction of vortices produced by the action of a single flagellum of an endodermal cell of brown hydra.



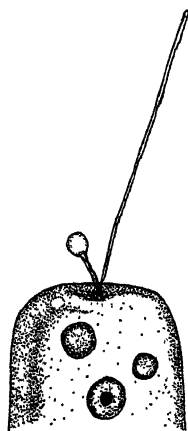
## PLATE 2

### EXPLANATION OF FIGURE

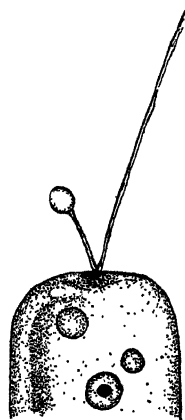
Diagram showing time intervals involved in flagellary formation on an uninjured endodermal surface.



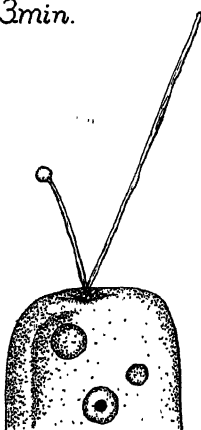
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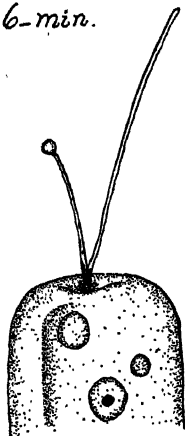
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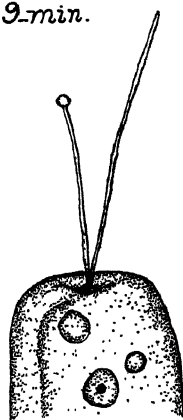
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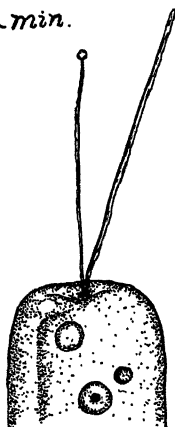
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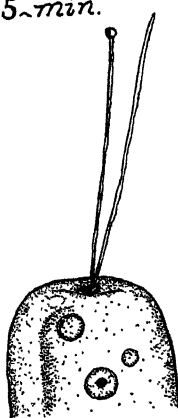
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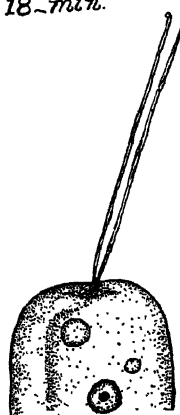
18-min.



21-min.



24-min.



27-min.



## PLATE 3

### EXPLANATION OF FIGURES

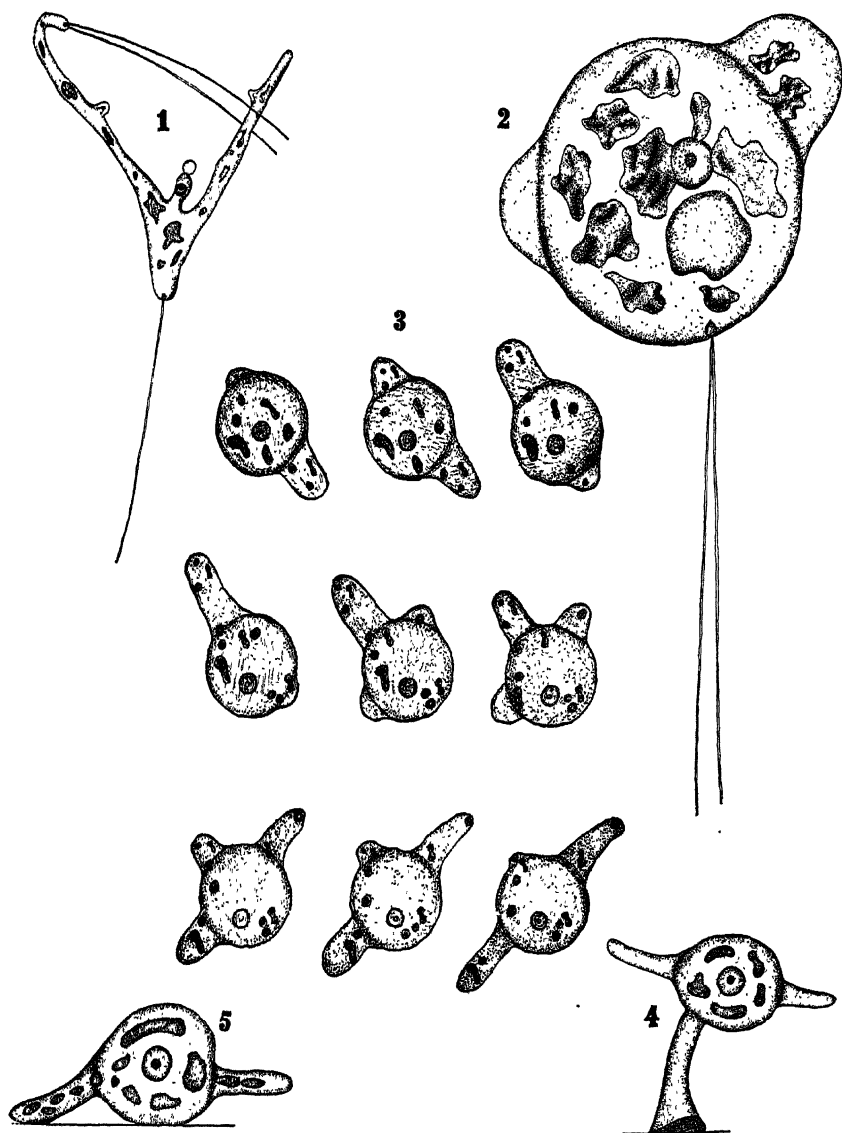
1 An isolated endodermal cell from the tentacle after having been separated from the mesoglea about four hours. Note two flagella arising from one blepharoplast; nucleus; excretion bubble; and odd shape of the cell. The flagella were very active and caused the cell to be moved violently about as the latter continuously changed form.

2 A typical isolated cell after having been separated from the mesoglea about two hours. Note flagella, irregular cytoplasmic inclusions, and pseudopods.

3 The pseudopods formed by an isolated endodermal cell over a period of nine minutes. Notice that the cytoplasmic inclusions flow into pseudopods as the latter are formed.

4 Drawing to show relation of pseudopods to substratum. A cell has arisen on a pseudopod from the surface of the slide, as if making an exploratory effort. In this position, they have been seen to radiate through a complete circle.

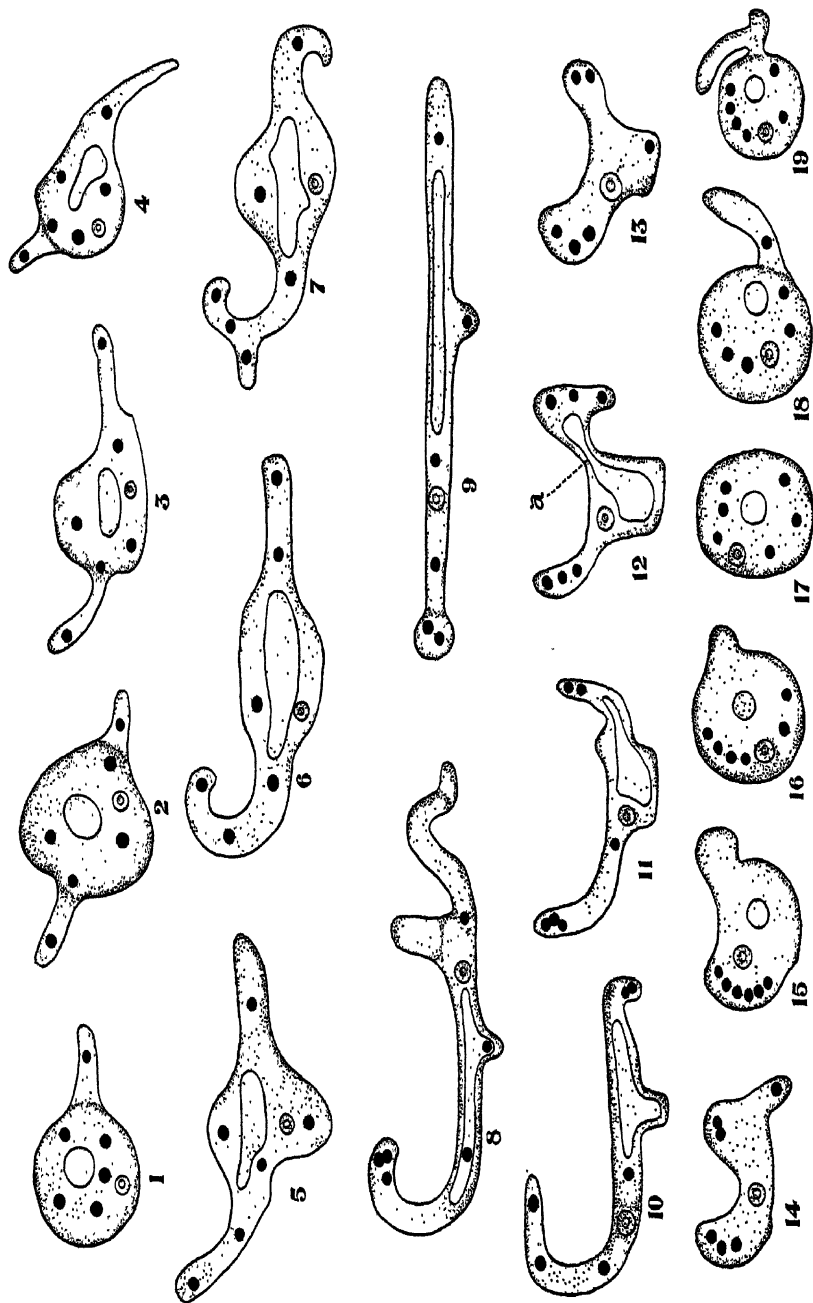
5 The same cell as in figure 4, after withdrawal of supporting pseudopod.



## PLATE 4

### EXPLANATION OF FIGURE

The forms assumed by an isolated endodermal cell over a period of three hours and ten minutes. At no. 12 vacuole burst through the cell membrane at *a*. At no. 15 another vacuole began to form. Green-hydra cell. Camera-lucida drawings.



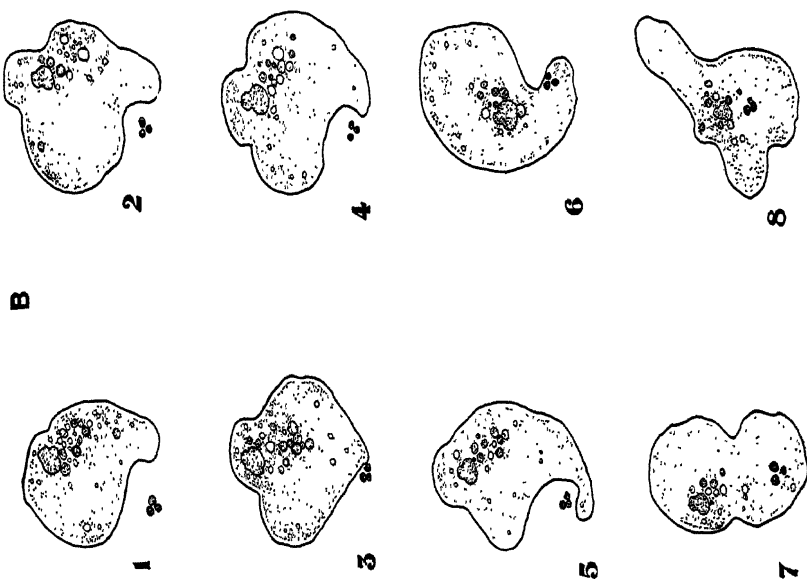
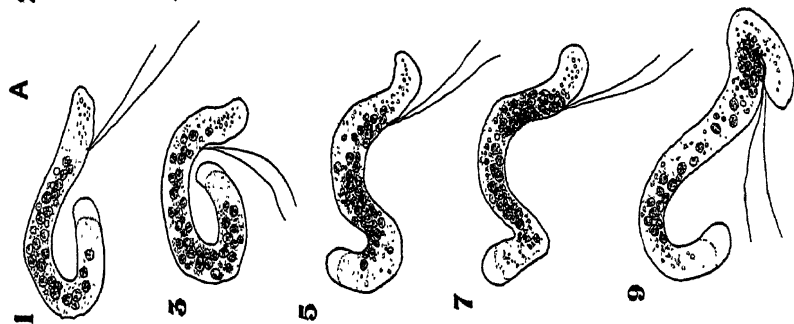
## PLATE 5

### EXPLANATION OF FIGURES

A An isolated endodermal cell of green hydra from middle third of body, showing forms assumed over a period of ten minutes. Note the clear end which has been fastened to the mesoglea. Drawings made from a series of photographs in the possession of the author.

B An isolated endodermal cell picking up particles of food. 1. Cell approaches food by means of a pseudopod. 2. Makes an effort as if to encircle food particle. 3. Upon coming in contact with food, cell withdraws pseudopod. 4. Cell again begins to encircle food particle with pseudopod. 5. Pseudopod very much extended. Immediately upon coming in contact with food particles, the cell takes them in. 6. Cell immediately changes its entire shape. 7 and 8. Cell begins moving off in another direction. Drawing made from photomicrographs taken at intervals of one minute.

ENDODERM OF HYDRA  
CARL H. MC CONNELL



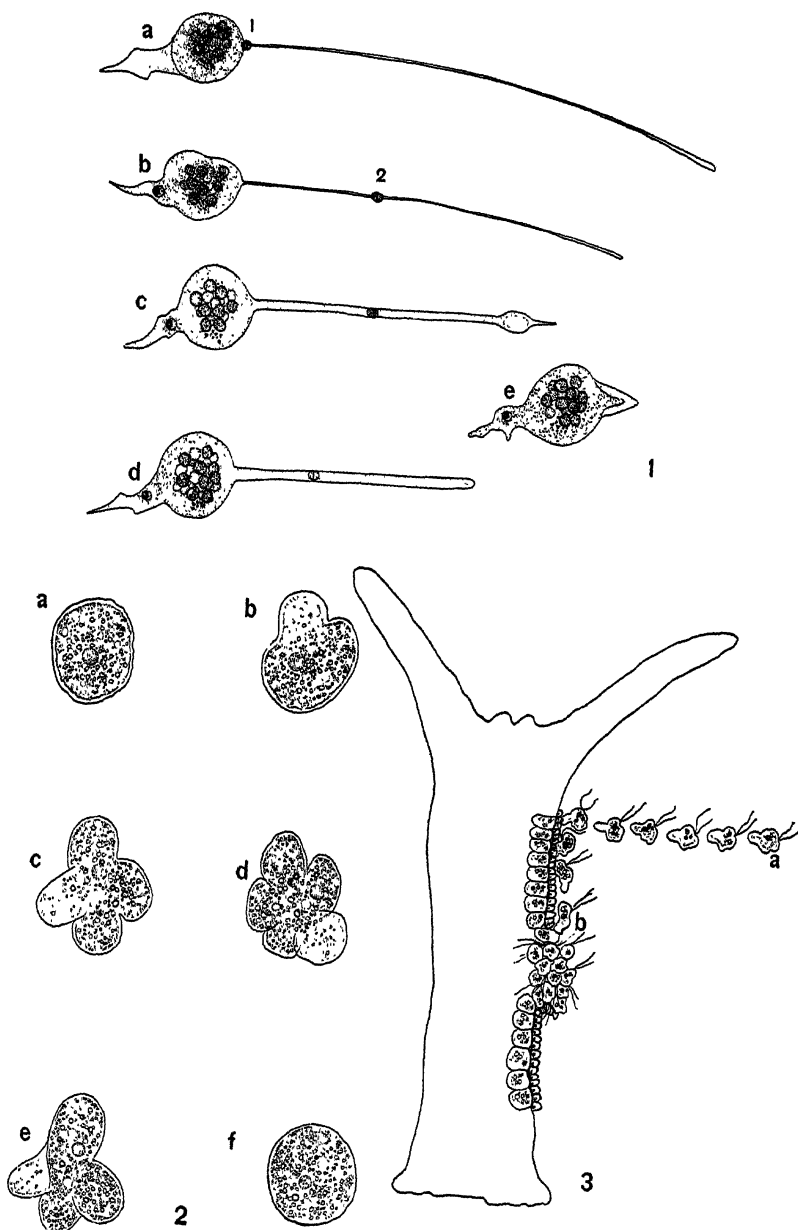
## PLATE 6

### EXPLANATION OF FIGURES

1 A pseudopod that presented no greater dimensions than a flagellum. A zoochlorella (1) left the cell body and was carried out to position 2 in *b*; meanwhile the pseudopod was shortening. The pseudopod continued to shorten, with the result that the zoochlorella and pseudopodial cytoplasm were withdrawn into the cell body (*c*, *d*, *e*). This series of observations extended over one minute and thirty seconds.

2 Forms assumed by an isolated endodermal cell of the basal disc from brown hydra, over a period of six minutes. Note that *a* and *f* are alike. This cell continued to exhibit these phenomena for two hours and forty minutes.

3 Diagram to show the distance (based upon its own dimensions) covered by an amoeboid cell in returning to an area where it coalesced with other endodermal cells. Upon coming in contact with the ectoderm, the cell first sent out an exploratory pseudopod, then it began traveling down the ectoderm until it reached a mass of outbulged endodermal cells, with which it immediately coalesced. The distance covered by the cell from *a* to *b* was  $196\mu$ , and the time required for the movement was one hour and ten minutes. The flagella were active at all times, but could not dislodge the pseudopods with which the cell moved. Outline of polyp greatly disproportionate.







# A DEMONSTRATION OF THE VACUOME AND THE GOLGI APPARATUS AS INDEPENDENT CYTOPLASMIC COMPONENTS IN THE FRESH EGGS OF TELEOSTEAN FISHES

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FIVE PLATES (TWENTY-NINE FIGURES)

## AUTHORS' ABSTRACT

The most important conclusion of this investigation is that the vacuome and the classical Golgi apparatus are independent cell components in the oocytes of *Ophiocephalus* and *Rita*. The same conclusion has been arrived at by Nath in *Rana tigrina*. The vacuoles, the Golgi elements, and the mitochondria can be seen *intra vitam* side by side.

In *Ophiocephalus* the vacuoles give rise to albuminous yolk, as has been rightly claimed by Hibbard for *Discoglossus* and by Hibbard and Parat for *Perca* and *Pygosteus*. The Golgi elements give rise to the fatty yolk as in so many other forms of oogenesis described by Nath and others.

In *Rita* the fate of the vacuoles and the Golgi elements is unknown, as the fish suddenly disappeared from the Ravi during the spawning period.

## INTRODUCTION

In 1926, the senior author described vesicular or vacuolar Golgi elements in the eggs of a spider, a chilopod, and the common firefly of Lahore. Each Golgi element was said to have a duplex structure consisting of an osmiophilic or argentophilic rim and an osmiophobic or argentophobic central substance—a form exactly similar to that of the Golgi elements of some Protozoa and the female germ cells of *Ascaris* previously described by Hirschler ('14) and many others in different kinds of cells. Parat had recently ('25, etc.) announced his vacuome theory, according to which the classical osmiophilic or argentophilic Golgi material is identical with the neutral-red-staining vacuoles of the cell. The vacuolar nature both of Parat's vacuome and the Golgi elements in eggs which appear in the form of vesicles impressed the senior author and he attempted to coordinate

Parat's conclusions with his own ('26 a). But further work carried out in this laboratory on the eggs mentioned above and many others showed that the vacuome is chemically and functionally a fundamentally different material from the classical Golgi apparatus which may very often appear in eggs and other cells in the form of vesicles. It was discovered that, whereas the vacuome stained brilliantly with neutral red according to Parat, the Golgi vesicles failed to stain in the eggs of the spider (Nath, '28), the chilopod (Nath and Husain, '28), the firefly (Nath and Mehta, '29), the culicine mosquito (Nath, '29), the Indian earthworm (Nath, '30), the common Indian frog (Nath, '31, *Z. Zellforsch.*), the medicinal leech (Nath, unpublished), a fresh-water crab and a prawn (Nath and D. R. Bhatia, unpublished), and the teleostean fishes which form the subject matter of the present investigation. Only in the cockroach (Nath and Piare Mohan, '29) the central substance of the Golgi vesicle may be 'very slightly' stained, but the cortex of the vesicle stands out as an unstained dark-grayish and refractile material. Bowen ('28 b), after reviewing the whole literature on the subject, also comes to the conclusion that the Golgi apparatus cannot be stained with neutral red, and where it is reported to have stained it has been confused with the vacuome.

The difference between the vacuome and the true Golgi vesicles became still more apparent when Hibbard ('28) and Hibbard and Parat ('27 and '28) claimed that in the eggs of the amphibian *Discoglossus* and the teleosteans *Perca* and *Pygosteus*, respectively, the Golgi apparatus (vacuome) gives rise to the albuminous yolk—a view which is basically different from the now well-established view of Nath that the classical lipoidal or fat-like Golgi vesicles give rise by a process of swelling to the fatty yolk. (See Nath, '30, for details of this theory.) Recently, the oogenesis of the saw-fly has been independently worked out in Professor Peacock's laboratory by Gresson ('29), who has confirmed in every detail the origin of fatty yolk vacuoles from the vesicular Golgi elements. Similarly, King ('26) described most circumstantially that in

*Oniscus asellus* the Golgi elements are directly metamorphosed into the fatty yolk vacuoles by a process which is strictly comparable to that of *Lithobius* as described by Nath ('24) and to that of *Helix aspersa* as described by Brambell ('24).

Since the publication of Parat's and Hibbard's papers it became evident that they are dealing with a cell component (vacuome) which is chemically and functionally very different from the classical Golgi material of a lipoidal (fat-like) nature. These differences have been emphasized in two recent publications from this laboratory (Nath, '30, and Bhandari and Nath, '30). Hibbard has recognized in the egg of *Discoglossus* only two primary cytoplasmic components, namely, the vacuome and the mitochondria. In addition, she has described fat globules. Similarly, Hibbard and Parat have described the vacuome and the mitochondria only in the oocytes of *Perca*, and in those of *Pygosteus* they have described oil droplets also. In other words, according to Parat and Hibbard, the classical lipoidal Golgi apparatus is a myth which results from the excessive precipitation of metallic osmium and silver on the surface and in the interior of the non-lipoidal and 'acidic' aqueous vacuoles.

Bearing in mind the very important issues involved in this controversy, it was decided to work out in this laboratory in the greatest possible detail the oogenesis of the common Indian frog *Rana tigrina* and that of the teleosts *Ophiocephalus punctatus* and *Rita rita*, as it appeared from the descriptions of Parat and Hibbard that the amphibian and the fish oocytes were very favorable for the study of the vacuome. We have made an extensive and repeated study of the fresh oocytes and have prepared slides with the current technique merely for the purposes of control. For in *Rana* and *Rita* the non-lipoidal, acidic, and neutral-red-staining vacuome, the classical osmiophilic or argentophilic lipoidal Golgi elements, and the mitochondria (lipoproteins) can be observed side by side in the living oocytes even without the aid of neutral red or osmic acid, with an ease which is simply

astounding. In *Ophiocephalus* the mitochondria and the vacuome can be similarly observed. The Golgi elements in the youngest oocytes, on the other hand, can be demonstrated only after brief periods of osmication, but in advanced oocytes where they have grown in size and become fatty they are visible intravital.

In the course of the preparation of the manuscript of this paper we have been very much gratified to read the letter of Professor Gatenby and Doctor O'Brien in *Nature* of June 14th. These authors also claim that in neutral-red-stained fresh preparations of the oocytes, the nerve cells, and the coelomic epithelial cells of *Lumbricus* the vacuoles, the Golgi elements, and the mitochondria can be seen side by side. Similarly, in the course of our investigations we read with satisfaction the recent letter of Professor Bhattacharya and Doctor Das ('29), who, in neutral-red-stained preparations of the young oocytes of pigeon, have demonstrated that the vacuoles and the classical Golgi apparatus are independent cell components. But the oocytes of *R. tigrina*, *Rita*, and *Ophiocephalus* are very much more favorable material for such a demonstration, because the vacuoles, on account of the density of their contents, can be seen intravital without the aid of neutral red as whitish droplets along with the Golgi elements and the mitochondria.

The demonstration of the vacuome and the Golgi elements as independent cell components in the egg cells completes a long series of such demonstrations in the plant cells, the male germ cells, and the somatic cells. At first Bowen ('27 and '28) demonstrated in several kinds of plant cells, including representatives of both germinal and somatic tissues from the Bryophyta, Pteridophyta, and Spermatophyta, the existence of osmiophilic platelets which, to say the least, much more nearly conform to the classical lipoidal Golgi apparatus of the animal cell than the vacuome. Bowen's extensive researches on the plant cells have been recently confirmed by Patten, Scott, and Gatenby ('28). Similarly, Parat's 'lepidosome' theory in the animal male germ cells can no longer be

upheld in the light of recent research. The work of Hirschler ('27, '27 a, '28, and '28 a), Monné ('27), Voinov ('27), and Gatenby ('29) on the male germ cells has very clearly shown that the so-called 'lepidosomes' are the classical lipoidal Golgi elements which are consistently argentophil, whereas the vacuome is not. For a full discussion of the evidence against the 'lepidosome' theory reference may be made to the very remarkable paper of Gatenby. In animal somatic cells, also, Beams ('30) has shown that the neutral-red-staining vacuome is not the Golgi material which is represented by the classical osmiophilic network.

The available evidence warrants the statement that the Golgi elements, like the mitochondria, are polymorphic. They may be granular or vesicular, e.g., in many eggs (Nath, King, Gresson, etc.) and Protozoa (Hirschler, '27); rod-like or crescent-shaped, e.g., in male germ cells (Bowen, Gatenby, etc.); reticular, e.g., in gland cells (Bowen, Beams, etc.), and, lastly, in the form of platelets as in the plant cells (Bowen, etc.). The root cause of the ephemeral popularity of the vacuome theory is the very important fact that the classical lipoidal Golgi apparatus does exist in many cases in the form of vesicles which in their hollow nature resemble the vacuome. But chemically and functionally they are diametrically opposite. The Golgi elements are not stainable with neutral red and are violently osmiophilic and argentophilic, whereas the vacuoles do not show the least sign of blackening, however heavy the impregnations, and are stainable with that vital dye. That is not all. The resemblance between their vacuolar nature is only superficial. The vacuole is merely a drop-let of a non-lipoidal, non-fatty, and aqueous material which in sections appears as a clear empty space, while the Golgi vesicle is surrounded by a thick lipoidal cortex.

The technique is mentioned in the text, but it seems necessary here to describe briefly the manner in which neutral red has been used. Thick watery solutions of the dye are injected into the body cavity of the fish. In a few hours not only the ovaries, but even the muscles of the body wall may take up

the stain. It was discovered that only the oocytes lying in the outer regions of the ovary could be thus stained, the stain failing to reach the inner oocytes. To insure more rapid penetration small pieces of the ovary were transferred to normal saline in a watch-glass to which are added two or three drops of the thick watery solution of the dye. The vacuoles stain light pink, but both the mitochondria and the Golgi elements are not affected at all by the stain.

We also find it necessary to describe certain experiments that we have performed with formalin-silver nitrate fixation (Da Fano) in the case of *Ophiocephalus*. We possess five sets of Da Fano slides of this material. In all sets the initial fixation in formalin-cobalt nitrate mixture was done for six hours. But the time of immersion in silver nitrate and the reducing mixture was made to vary. In one set the time of immersion in silver nitrate was forty-eight hours and the reduction was allowed for about sixteen hours. In the other set silver nitrate was allowed to act for four hours and the reducing mixture for one hour and twenty minutes only. The other three sets were intermediate between these two extremes with respect to the periods allowed in silver nitrate and the reducing mixture. The most interesting feature of these experiments is that in all the five sets of slides the Golgi elements invariably went jet-black, whereas the vacuoles did not show the least amount of blackening.

#### OBSERVATIONS

##### *Ophiocephalus punctatus*

a. *Vacuome and albuminous yolk.* When a young oocyte (fig. 1) of *Ophiocephalus* is studied in a drop of normal saline, the most prominent inclusions are certain whitish droplets distributed at random in the cytoplasm. These have been identified as the vacuome of Parat and Hibbard. They are stainable with neutral red. As a rule, they stain uniformly with this dye, appearing as light pink droplets (fig. 2), but sometimes the stained contents of a droplet may shrink and appear as a red grain lying in the center of a clear circular

area (fig. 3), reminding us of the same phenomenon in *Rana tigrina* and of the figures of the plant vacuome recently published by Guilliermond ('29). Although in some fixed preparations the droplets may coalesce and thus give rise to irregular canals, etc. (e.g., fig. 7), such a phenomenon is very rare indeed in the fresh material. At one place at least in the bigger oocyte in figure 3 coalescence of vacuoles seems to have taken place.

In fixed preparations of young oocytes these droplets appear in the form of clear circular spaces many of which show a coagulum inside them. The most interesting feature of the vacuoles is that they may show a coagulum inside them, even in the youngest oocytes (fig. 4). In other words, their contents are dense. This explains the extraordinary ease with which they can be seen in the fresh oocytes even without the aid of neutral red. In Champy unstained preparations the contents of the vacuoles are yellowish (figs. 4 to 8) and the same is true of Kolatchev unstained preparations (fig. 9). In Da Fano preparations the coagulum fails to appear, the vacuoles appearing as empty spaces (figs. 13, 15, and 16). In Bouin iron-haematoxylin preparations the coagulum is very prominent and stains gray to blue-black (fig. 10).

Since the coagulum inside the vacuoles appears excellently in Bouin's preparations, it is evident that the contents of these vacuoles are albuminous in nature. Add to this the facts that the vacuoles do not show the least amount of blackening in Da Fano (figs. 13, 15, and 16) and Kolatchev (fig. 9), however heavy the impregnations, that both in Champy unstained (figs. 4 to 8) and fresh cover-slip preparations osmicated for brief periods (figs. 11 and 12) they do not go black, and the conclusion becomes irresistible that the contents of the vacuoles are non-lipoidal and non-fatty.

In the earliest oocytes the vacuoles are small and comparatively delicate. In the course of oogenesis they grow in size and their contents become denser as the result of the deposition of albuminous material inside their interior. Consequently, when an oocyte measuring about 0.2 mm. (fig. 17) is



ruptured, the vacuoles which are now packed with albuminous material show a firm consistency and retain their rounded form. The contents of the vacuoles continue to become denser, till ultimately in the 'ripe' egg (fig. 19) the metamorphosis of the small delicate aqueous vacuoles into the semi-solid albuminous yolk bodies is completed.

*b. Golgi apparatus, fatty yolk, and mitochondria.* When the oocyte measures about 0.08 mm., a constant feature is the presence of a dense circumnuclear ring. It consists of the Golgi and mitochondrial granules, but very often it contains the vacuoles also. In the absolutely fresh preparations (fig. 1) the Golgi elements cannot be seen, but the mitochondria appear as very small white-grayish granules densely packed together. If, however, the oocytes are osmicated for forty-eight hours, the Golgi elements appear as dark grains lying in the circumnuclear ring (fig. 11). They are intermediate in size between the mitochondrial granules and the vacuoles, both of which appear yellowish as the result of osmication. If the osmication is prolonged to six days at room temperature, some of the Golgi elements appear as very tiny vesicles, each vesicle showing a dark refractile cortex and a central clear area (fig. 12). Similarly, in Champy unstained preparations of the oocytes of this stage (studied immediately after mounting) the Golgi elements and the mitochondria appear as black and yellowish grains, respectively, and the vacuoles as clear spaces with or without the yellowish coagulum (fig. 7). In Kolatchev (fig. 9) appearances are the same as in Champy except that the Golgi grains are much more black. In Da Fano (fig. 13) the Golgi elements are jet-black, the mitochondria are gray to golden, and the vacuoles appear as clear empty spaces.

In the earliest oocytes we have not been able to identify the mitochondria (figs. 4 and 14). Even Champy preparations stained with iron-haematoxylin do not demonstrate them. But as soon as the oocyte measures about 0.08 mm. they can be observed, even in the fresh cover-slip preparations, arranged in a circumnuclear fashion like the Golgi elements.

The characteristic circumnuclear ring consisting of the mitochondria, the Golgi elements, and also many, if not all, the vacuoles, gradually extends toward the periphery of the egg without undergoing fragmentation (fig. 15). The result of this peripheral extension is that when the oocyte measures about 0.2 mm. all the three inclusions are more or less uniformly dispersed throughout the cytoplasm (figs. 8 and 16).

When an oocyte measuring about 0.2 mm. is ruptured and its contents studied, the first thing that one notices is that the Golgi elements which could not be observed in the younger fresh oocytes now stand out prominently as dark refractile grayish granules contrasting violently with the vacuoles, which have now become much denser and therefore look more yellowish than whitish (fig. 17). In such preparations the mitochondrial granules cannot be observed, on account of their very small size, but in Champy and Da Fano preparations of oocytes of the same age they can be distinctly made out (figs. 8 and 16).

If entire oocytes measuring 0.2 mm. are studied after osmication (from twenty-one hours to six days) at room temperature, the Golgi elements which appear granular in the fresh preparations now show their duplex structure (fig. 18). Each element appears as a tiny vesicle with a dark refractile cortex and a very slightly brownish central area. A comparison of figures 11 and 17 clearly shows that the Golgi elements grow in the course of oogenesis. It further shows that, whereas in younger oocytes (fig. 11) the Golgi elements cannot be demonstrated unless they are osmicated for at least forty-eight hours, they stand out prominently in the fresh oocytes measuring about 0.2 mm. as dark, refractile, and grayish granules (fig. 17). After an osmication of twenty-one hours (fig. 18), they appear darker and clearly show their vesicular nature. From all this one has to conclude that the Golgi elements not only grow in the course of oogenesis, but become slightly fatty also.

Throughout the subsequent stages of oogenesis many Golgi elements continue to grow and become more and more fatty.

If a very advanced oocyte measuring 0.97 mm. is ruptured after only ten minutes' osmication and its contents studied under the microscope, one is driven to the conclusion that the small Golgi vesicles give rise to fatty yolk by a process of growth and deposition of fat inside their interior (fig. 19). The striated spheres represent the albuminous yolk which is condensed inside the vacuoles. These appear slightly yellowish. Then there are dark vesicles of different sizes showing different grades of black.

Advanced oocytes are fixed in formalin (formalin, 10 cc.; H<sub>2</sub>O, 50 cc.) overnight. In the morning they are transferred to an alcoholic solution of sudan III, in which they are kept for fifteen minutes only. They are then ruptured and their contents are studied under the microscope. The fatty yolk goes deep orange and the albuminous yolk is not affected at all.

#### *Rita rita*

The ovaries of *Rita* were constantly studied throughout the winter, but the oocytes were found to be devoid of yolk. From the month of October till April, the oocytes never showed any yolk and they all measured less than about 0.5 mm. During the spawning season, which is the month of May, they suddenly disappeared from the Ravi and our best efforts to obtain them only brought on us the ridicule of the fishermen. On the other hand, the ovaries of *Ophiocephalus* throughout winter contained oocytes which measured 0.25 mm. only and yet contained albuminous yolk inside them (fig. 10). The oocytes of *Ophiocephalus* never measured more than 0.25 mm. in winter, till in the month of April 'ripe' eggs measuring about 1 mm. and containing both the albuminous and fatty yolk were found.

Our investigations on *Rita* oogenesis, therefore, could extend only up to the stage when the oocytes measure about 0.5 mm. But that does not in any way detract from the value of this material for the object we have in view, namely, the demonstration of the vacuome and the Golgi elements as independent cytoplasmic components. Indeed, *Rita* oocytes excel

in this respect those of *Ophiocephalus* and even *Rana tigrina*, because the Golgi elements of this species are very highly refractile and can be seen in the fresh material with an ease which is simply astounding.

When an oocyte of *Rita* measuring about 0.4 mm. is studied in a drop of normal saline, the Golgi elements are seen arranged in groups and confined to the periphery only (fig. 20). They are in the form of very highly refractile granules with a very definite bluish tinge. The vacuoles, on the other hand, are whitish and are distributed uniformly, the bigger ones confined to the inner regions of the cytoplasm. The white-grayish mitochondrial granules are extremely small and are, like the vacuoles, uniformly distributed. In figure 21 is shown a portion of the same oocyte seen in the uppermost plane, the nucleus not appearing, as it lies in the middle plane of the oocyte. In figure 22 is drawn a portion of an oocyte measuring about 0.5 mm. as seen in the uppermost plane. The nucleus can be seen because it has migrated to the periphery of the oocyte. The groups of Golgi elements are bigger, indicating that the elements are proliferating.

The youngest oocyte in which we have seen the Golgi elements in the fresh material is shown in figure 23. Here the Golgi elements are juxtannuclear and the vacuoles more or less circumnuclear. The latter mask the fine mitochondrial granulation. In the next stage all the three cytoplasmic inclusions begin to disperse (fig. 24), till finally the vacuoles and the mitochondria get uniformly distributed, and the Golgi elements migrate to the periphery.

The material that we have described under the name of Golgi reacts to the Mann-Kopsch technique like the classical Golgi apparatus. It is difficult to impregnate it, but it goes jet-black after thirty-two days of immersion in osmic acid at room temperature. It does not show the least amount of blackening after Champy and partially retains its characteristic bluish tinge in such preparations. Repeated attempts to impregnate it in Kolatchev proved infructuous, but it went black in Da Fano.

In the earliest oocytes that we have been able to study in the fixed preparations the Golgi grains have a very constant juxtannuclear disposition. Whereas the Golgi grains are intensely blackened, the vacuoles do not show the least amount of blackening. They appear as empty spaces without any coagulum (fig. 25). A number of vacuoles may coalesce artificially and give rise to irregular canals (compare figs. 25 and 29). Gradually the juxtannuclear Golgi apparatus spreads out in the cytoplasm till ultimately it migrates to the periphery (figs. 26 to 28). In Bouin iron-haematoxylin preparations the vacuoles appear as empty spaces and the Golgi elements are completely washed out. But the mitochondria persist as highly corroded grayish granules.

#### DISCUSSION

##### *a. Vacuome and albuminous yolk*

One thing that emerges clearly from the present investigation is that in the eggs of both *Ophiocephalus* and *Rita* the vacuolar system and the Golgi apparatus are independent cell components. We emphasize the most important fact that in both these forms the Golgi elements, the vacuoles, and the mitochondria can be seen side by side in the fresh material. The same conclusion has been arrived at by Nath in *Rana tigrina*, by Bhattacharya and Das in the pigeon, and by Gatenby and O'Brien in the British earthworm. In the earthworm ovary the vacuolar system has been demonstrated after treatment with neutral red for about an hour. Similarly, in the pigeon ovary treatment with neutral red for about twenty minutes has been found necessary for the demonstration of the vacuome.

In *Ophiocephalus* and *Rita*, on the other hand, the vacuoles can be seen as whitish refractile droplets in the earliest oocytes in the absolutely fresh material without the aid of any vital dye. The same is true of the vacuolar system of *R. tigrina* when the oocyte measures about 0.45 mm. This extraordinary ease with which the vacuoles can be seen *intra-vitam* in the oocytes of these three species is due to the

greater density of their contents. In the earthworm oogenesis yolk is not elaborated and therefore the vacuoles remain delicate and cannot be seen intravital. In the pigeon, also, the vacuoles have been demonstrated in the very young oocytes with the aid of neutral red, and we await with interest the full account of Bhattacharya and Das to know whether the contents of the vacuoles become dense or not in the course of oogenesis.

In *Ophiocephalus* the contents of the vacuoles are so dense even in the earliest oocytes that a yellowish coagulum appears very distinctly inside them in Champy and Kolatchev unstained preparations. In fresh cover-slip preparations stained with neutral red the contents may contract and appear in the form of a red grain. In the course of oogenesis the contents of the vacuoles continue to become denser till ultimately the vacuoles form the albuminous yolk *sensu strictu*, as has been very rightly claimed by Hibbard and Parat for *Perca* and *Pygosteus* and by Hibbard for *Discoglossus*. We desire to emphasize this very important conclusion arrived at by the Parat school, and we gladly accept the view that the egg vacuome is strictly comparable with the plant vacuome in the interior of which also protein material in the form of aleurone grains is deposited.

In *Rita* the fate of the vacuome is unknown, as yolk-laden oocytes have not so far been available. Whether the vacuoles of *Rita* remain as such as in *R. tigrina* or whether they give rise to albuminous yolk as in *Ophiocephalus*, *Perca*, *Pygosteus*, and *Discoglossus* must await further investigation during the spawning season.

We are indebted to the Parat school not only for their focusing attention on a hitherto neglected cell component, namely, the vacuome, but also for demonstrating that it may give rise in oogenesis to albuminous yolk. But the vacuome does not always give rise to albuminous yolk. In *R. tigrina* the contents of the vacuoles do become somewhat dense, but in all sections they appear as clear empty spaces up to the end of oogenesis. Nath has shown that the yolk of the egg of

*R. tigrina* comes in an unmistakable manner from the mitochondria, as had been previously claimed by Lams ('07) and Konopacki ('27) for some other species of *Rana*, by Gajewska ('15) for *Triton*, and by King ('26) for *Oniscus*. In other forms of oogenesis albuminous yolk undoubtedly arises from nucleolar extrusions, e.g., *Saccocirrus*, cockroach, *Lithobius*, *Luciola*, *Euscorpius*, *Buthus*, and *Dysdercus*, etc. (for references, see Bhandari and Nath, '30). Most recently, Gresson ('29 and '29 a), working on the saw-flies, has confirmed the nucleolar origin of protein yolk. In still other cases such yolk may arise *de novo* in the cytoplasm, as, for example, in the spider egg (Nath, '28).

If there are no nucleolar extrusions in the fish egg, it must not be inferred that the nucleus does not take part in vitellogenesis. In both *Ophiocephalus* and *Rita* and also in *R. tigrina* there is a single nucleolus in the earliest oocytes. In the course of oogenesis a large number of nucleoli appear, but no nucleolar buds are extruded into the cytoplasm, and none have been described by any previous worker either in the frog or in the fish. But the enormous increase in the size of the nucleus and the multiplication of nucleoli seem to indicate that the nucleus is controlling in some manner the process of vitellogenesis. It may be that some material which cannot be detected by the microscope passes from the nucleus into the cytoplasm.

#### *b. Golgi apparatus and fatty yolk*

In *Rita* the Golgi elements which are in the form of granules have a juxtannuclear disposition in the earliest oocytes. Gradually they spread out in the cytoplasm and eventually migrate toward the periphery of the oocyte, where they give strong indications of proliferation. They have a very characteristic bluish tinge and can be easily observed *intravital* along with the whitish vacuoles and the white-grayish mitochondria. The fate of the Golgi elements is unknown, like that of the vacuoles. Whether they remain as such throughout oogenesis, as in the culicine mosquito (Nath, '29), the

earthworm (Gatenby and Nath, '26, and Nath, '30), and the frog (Nath, '31), or whether they swell up and give rise to fatty yolk, as in *Ophiocephalus*, *Lithobius*, *Helix*, *Oniscus*, spider, the chilopod *Otostigmus*, the firefly *Luciola*, the cockroach, and the saw-flies, must await further investigation during the spawning season.

In *Ophiocephalus*, on the other hand, the Golgi grains in the youngest oocytes have a circumnuclear disposition. They have in such oocytes a low refractive index and can be observed in the fresh cover-slip preparations after forty-eight hours' osmication only. Very rarely, however, they may appear in neutral-red-stained preparations as dark grayish granules contrasting with the pinkish vacuoles. In the course of oogenesis the Golgi grains grow into tiny vesicles which can be observed intravital when the oocyte measures about 0.2 mm. Toward the approach of the spawning season the Golgi vesicles grow enormously in size and give rise to the fatty yolk vacuoles. The whole evidence in favor of the Golgi origin of fatty yolk in general has been set forth in detail recently by Nath ('30) and by Bhandari and Nath ('30), and here we content ourselves by emphasizing the fact that in 'ripe' eggs of *Ophiocephalus* osmicated for five to ten minutes there are all gradations between the fatty yolk vacuoles and the lipoidal Golgi elements.

We once more emphasize the most important fact that in *Ophiocephalus*, *Rita*, *Rana tigrina*, and the earthworm the vacuoles, the Golgi elements, and the mitochondria can be seen side by side in the fresh material. How will, then, the exponents of the vacuome theory interpret this material which we have described under the name of Golgi? It cannot be interpreted as globules of fat. One of the tests of fat is that it quickly goes black in osmic acid. Gatenby ('21), for example, has suggested that anything that goes black in Champy and is subsequently decolorized by turpentine may be considered as fat. While fats do undoubtedly go black in Champy or in osmic acid alone, the time has come when it must be recognized that the lipoidal classical Golgi appa-



tus also may go black in short periods of osmication. After all, we know that lipoids are fat-like in some respects. Bowen ('19 and '28 a) succeeded in blackening the classical Golgi apparatus in the hemipteran testis and other insects after twenty-four hours' immersion in Mann's corrosive-osmic mixture. Weigl ('10, as quoted by Bowen) found that after five to ten minutes' exposure to 2 per cent osmic acid at 25°C. some traces of the Golgi apparatus (presumably in somatic cells) were just barely visible. After one hour the blackening is more obvious. It will be preposterous to consider this material as fat merely because it can be blackened after short periods of osmication.

Nor are we justified to regard the Golgi elements of *Ophiocephalus*, *R. tigrina*, *Culex* (Nath, '29), and the earthworm as globules of fat simply because they go black in Champy after twenty-four hours. Nor again can we regard the Golgi elements of *Luciola* (Nath and Mehta, '28) and *Dysdercus* (Bhandari and Nath, '30) as fat simply because they go black in 2 per cent osmic acid in ten minutes. Mere fat cannot go black in Da Fano, whereas the Golgi elements which can also be blackened in Champy do, like the classical Golgi apparatus of nerve cells. They also go jet-black in Mann-Kopsch or Kolatchev and cannot be subsequently decolorized with turpentine, whereas blackened fat can be easily decolorized. Lastly, in *Rita* the Golgi elements which are blackened in Mann-Kopsch like the classical Golgi apparatus of nerve cells do not go black in Champy at all. As a matter of fact, they cannot be blackened even in Mann-Kopsch in less than thirty-two days, and with Kolatchev we have repeatedly failed to blacken them at all.

But we do not depend on the above reactions only for identifying the material under discussion as Golgi. Above all we have to consider the more important facts of its disposition and dispersal. If one bears in mind its juxtannuclear disposition in the earliest oocytes of *Rita*, *Rana*, and the earthworm, its circumnuclear disposition in *Ophiocephalus*, the cockroach, and *Dysdercus*, its subsequent dispersal in an

orderly fashion, which is remarkably orderly indeed in Rita and Rana, and lastly its existence in the tiny undifferentiated primordial cells of *Luciola* and Rana, it becomes impossible to entertain the suggestion that it represents inert globules of oil.

In their chemistry the vacuome and the Golgi apparatus are diametrically opposite. Whereas the Golgi elements go jet-black in Da Fano, Kolatchev, or Mann-Kopsch and are stainable with neutral red, the vacuoles do not show the least sign of blackening and are not affected at all by this vital dye. The vacuome therefore is non-lipoidal, as defined by Parat, whereas the Golgi apparatus is essentially lipoidal.

#### SUMMARY

1. The most important conclusion of this investigation is that the vacuome and the classical Golgi apparatus are independent cell components in the oocytes of *Ophiocephalus* and Rita. The same conclusion has been arrived at by Nath in *Rana tigrina*.

2. Were the above conclusion based on fixed preparations only, it would have been open to criticism. But such preparations have been used merely for the purposes of control. For luckily in Rita the bluish Golgi grains, the whitish vacuoles, and the white-grayish mitochondria can be seen intravital side by side. The same is true of *Ophiocephalus*, except that the Golgi elements are dark grayish.

3. The material described as Golgi apparatus is blackened in Da Fano, Kolatchev, and Mann-Kopsch like the classical Golgi apparatus of nerve cells, and is not stainable with neutral red. The vacuoles, on the other hand, are stainable with this dye, and do not show the least amount of blackening, however heavy the impregnations. The vacuoles therefore are non-lipoidal droplets of some aqueous material, whereas the Golgi apparatus is lipoidal. Strong evidence has been furnished that the material described under the name of Golgi does not represent inert globules of fat.

4. In *Ophiocephalus* the vacuoles condense inside them protein material and give rise to albuminous yolk, as has been very rightly claimed by Hibbard for *Discoglossus* and by Hibbard and Parat for *Perca* and *Pygosteus*. They are therefore strictly comparable with the plant vacuoles in which protein material in the form of aleurone grains is said to be condensed.

5. The Golgi elements of *Ophiocephalus* form the fatty yolk as in *Lithobius*, *Helix*, spider, *Otostigmus*, *Luciola*, cockroach, *Oniscus*, *Dysdercus*, saw-flies, etc.

6. In *Rita* the fate of the Golgi elements is unknown, as advanced oocytes have not been available. Whether they remain as such, as in the earthworm (Nath) and the culicine mosquito (Nath), or whether they form the fatty yolk must await further investigation.

The fate of the vacuoles is likewise unknown. Whether they remain as vacuoles, as in *Rana tigrina* (Nath in press), or form albuminous yolk, as in *Ophiocephalus*, etc., must also await further investigation.

#### ADDENDUM

After the manuscript of this paper had been sent to the press, the senior author received, through the courtesy of Mr. Dharam Narain, a reprint of his brief paper on the cytoplasmic inclusions in the oogenesis of *Ophiocephalus punctatus* (*Zeit. Zellforschg.*, Bd. 11, Heft 2, August, 1930). Throughout his account Mr. Dharam Narain has described only two categories of granules, namely, the mitochondria and the Golgi apparatus, but has made no mention of the vacuolar system as such—a system which is very prominent in the fish egg and which has been so admirably described by the Parat school. He has given a substantially correct account, we are glad to say, of the mitochondria and the classical lipoidal Golgi apparatus during the early stages of oogenesis (figs. 1 to 3 and 5 to 7), even though he failed to observe and describe the vacuome, in spite of his statement that intravital examination of material was also made. We are, however, compelled to say, not without considerable pain, that Mr. Dharam Narain has misinterpreted the inclusions when the oocytes measure about 0.2 mm. Nor is there any evidence in the paper of his having studied older oocytes.

Mr. Dharam Narain's conclusions are that the fatty yolk arises from the Golgi bodies and that the albuminous yolk appears to be formed directly or indirectly by the mitochondria. Although the first conclusion is identical with our own, we are compelled to state that he has not seen the fatty yolk vacuoles which appear in very advanced oocytes measuring about 1 mm. and which he never studied. The fact is that he has misinterpreted the vacuoles condensing albuminous material inside them as the fatty yolk vacuoles and the slightly swollen Golgi elements as the mitochondrial yolk (see his fig. 8 and compare it with our fig. 10).

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## PLATES

Figures 1 to 19, *Ophiocephalus*; figures 20 to 29, *Rita*. Further explanation of figures will be found in the text.

### ABBREVIATIONS

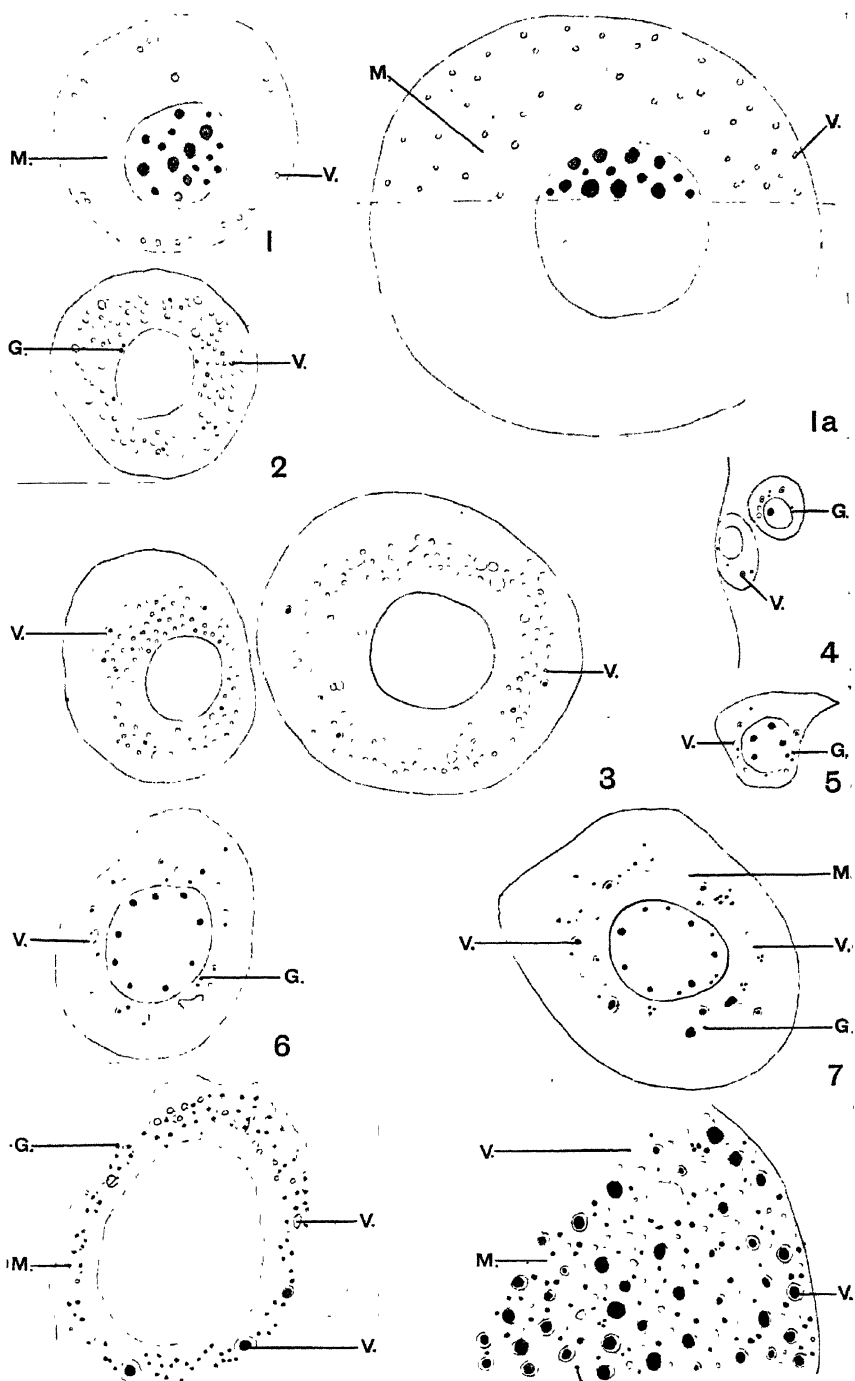
*A.*, albuminous yolk  
*F.*, fatty yolk  
*G.*, Golgi element

*M.*, mitochondria  
*V.*, vacuole

## PLATE 1

### EXPLANATION OF FIGURES

- 1 Fresh.  $\times 355$ .
- 1 a Fresh.  $\times 355$ .
- 2 Fresh. Studied three hours after neutral-red injection into body cavity.  
 $\times 355$ .
- 3 Fresh. Studied three hours after neutral-red injection into body cavity.  
 $\times 355$ .
- 4 to 8 Champy unstained. Studied immediately after mounting.  
4 to 7  $\times 450$ .
- 8 Portion of an oocyte measuring 0.2 mm.  $\times 355$ .
- 9 Kolatchev, unstained. Four days' incubation at 38°C.  $\times 799$ .

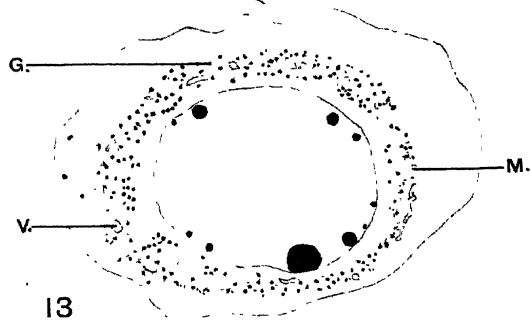
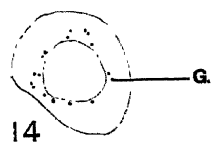
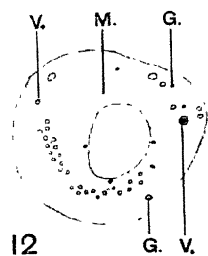
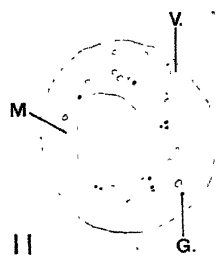




## PLATE 2

### EXPLANATION OF FIGURES

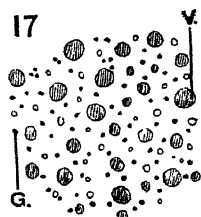
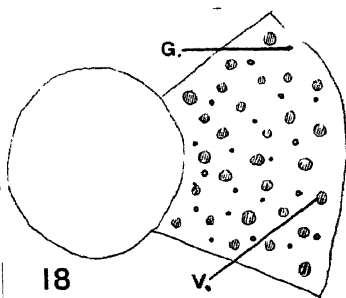
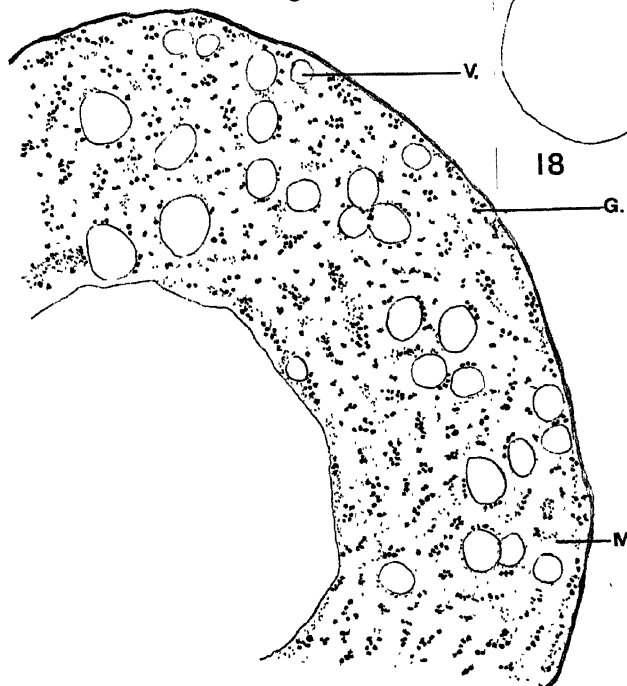
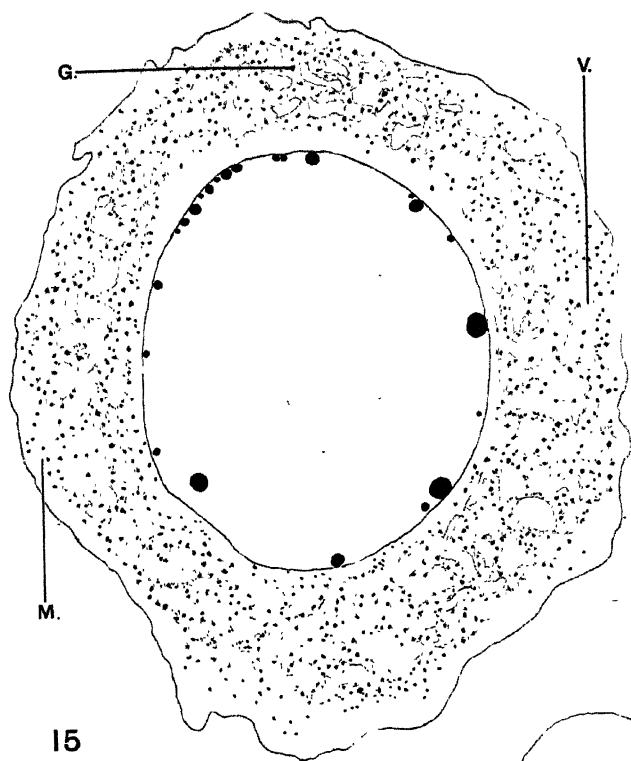
- 10 Bouin and iron-haematoxylin. Portion of an oocyte measuring 0.25 mm.  
× 710.
- 11 Fresh. Osmicated for forty-eight hours. × 355.
- 12 Fresh. Osmicated for six days. × 355.
- 13 Da Fano. Toned and stained with iron-haematoxylin. × 799.
- 14 Da Fano. Untoned. × 990.



### PLATE 3

#### EXPLANATION OF FIGURES

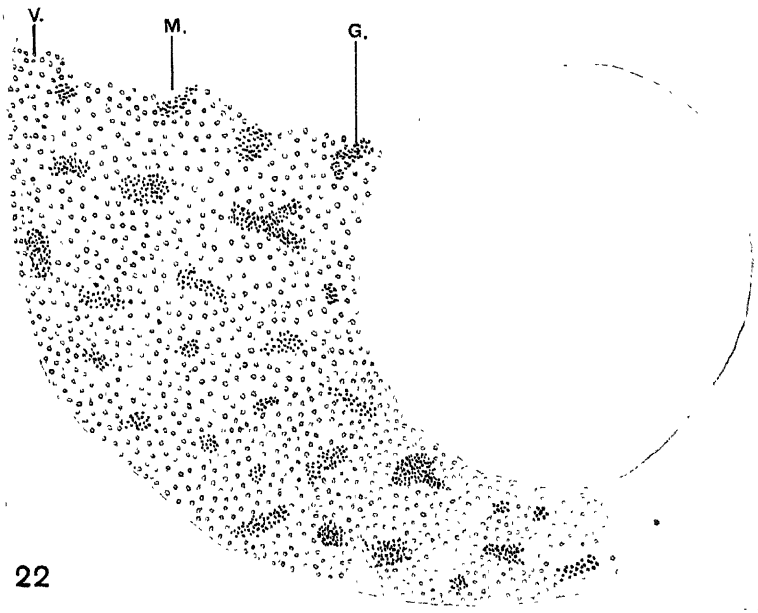
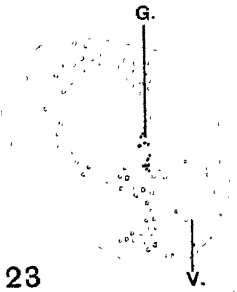
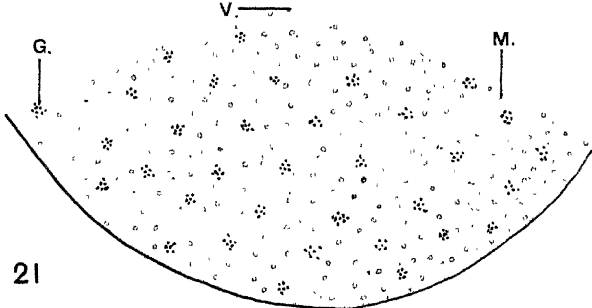
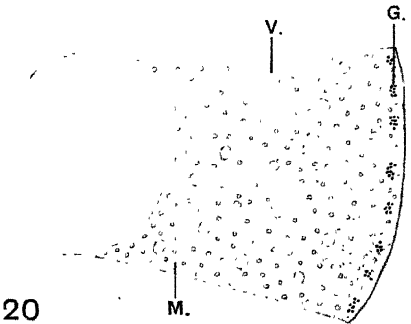
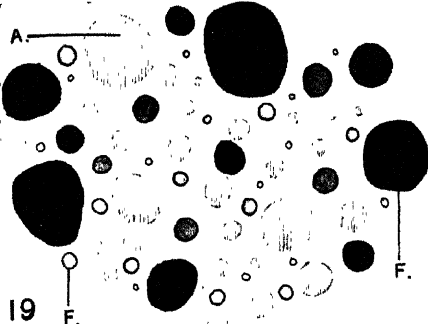
- 15 Da Fano. Toned and stained with iron-haematoxylin.  $\times 710$ .
- 16 Da Fano. Untoned. Portion of an oocyte measuring 0.2 mm.  $\times 799$ .
- 17 Fresh. Contents of an oocyte measuring 0.2 mm.  $\times 355$ .
- 18 Fresh. Osmicated for twenty-one hours. Portion of an oocyte measuring 0.2 mm.  $\times 355$ .



## PLATE 4

### EXPLANATION OF FIGURES

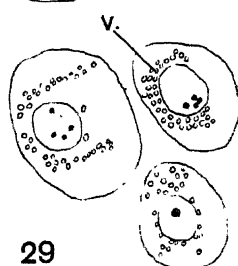
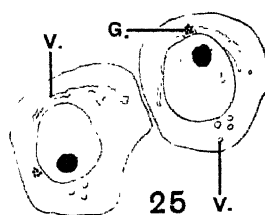
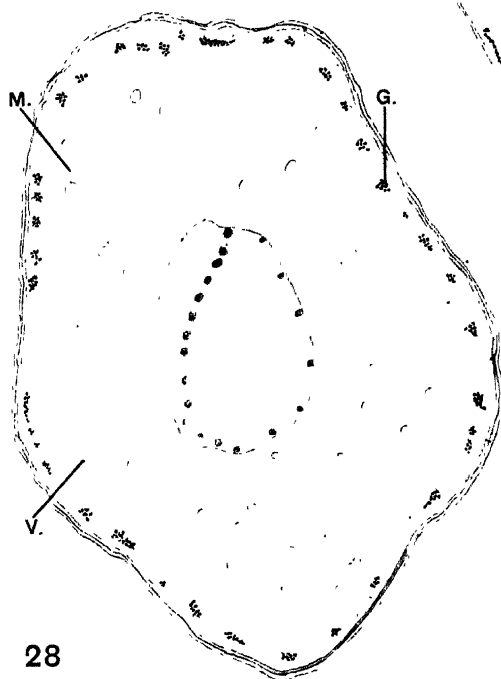
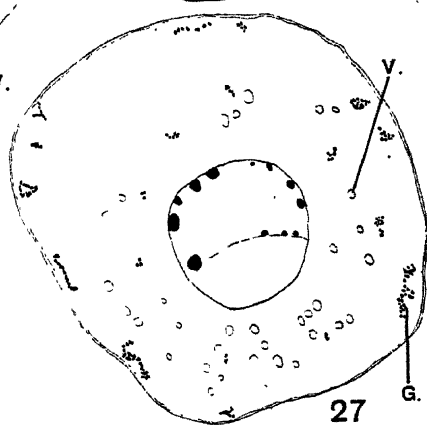
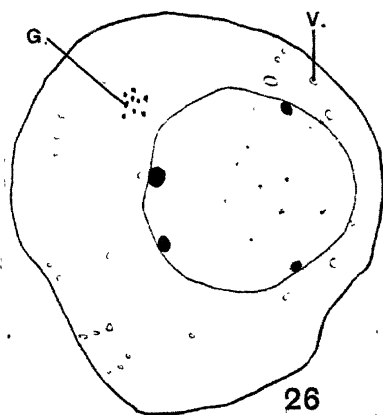
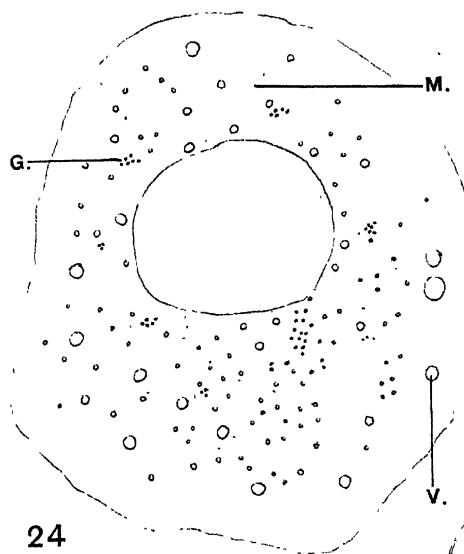
- 19 Fresh. Osmicated for ten minutes. Contents of an oocyte measuring 0.97 mm.  $\times 355$ .
- 20 Fresh. Portion of an oocyte measuring 0.4 mm., as seen in the middle plane.  $\times 355$ .
- 21 Fresh. Portion of the same oocyte as shown in figure 20. Surface view.
- 22 Fresh. Portion of an oocyte measuring 0.5 mm. Surface view.  $\times 355$ .
- 23 Fresh.  $\times 355$ .



## PLATE 5

### EXPLANATION OF FIGURES

- 24 Fresh.  $\times 355$ .
- 25 Mann-Kopsch.  $\times 710$ .
- 26 Mann-Kopsch.  $\times 710$ .
- 27 Mann-Kopsch.  $\times 355$ .
- 28 Mann-Kopsch.  $\times 355$ .
- 29 Fresh. Studied twenty-two hours after neutral-red injection into body cavity.  $\times 355$ .







# VERTEBRATE CEPHALOGENESIS

- VI. A. THE VELUM—ITS PART IN HEAD BUILDING—THE HYOID.  
THE VELATA. THE ORIGIN OF THE VERTEBRATE HEAD SKELETON  
B. MYXINOID CHARACTERS INHERITED BY THE TELEOSTOMI

HOWARD AYERS

TWENTY-TWO FIGURES

## AUTHOR'S ABSTRACT

The oldest parts of the fibrous, cartilaginous, and bony head skeleton of vertebrates descend from the amphioxid ancestors of the myxinoid fishes. The skeleton of the velum and jaw bars of the amphioxids transform into the jaw apparatus and hyoid-velar head skeleton of the myxinoids, and some parts are passed on by inheritance to the teleostome fishes (sturgeons). These anatomical characters prove descent with modification. The theory of the origin of jaws and hyoid from branchial cartilages is not supported by the facts, as both jaws and hyoid structures were developed long before branchial cartilages furnished material for head building. The Velata, the oldest of living vertebrates, include the amphioxids and marsipobranchs, and are marked off from the higher forms by the important organ, the velum, which separates the prebranchial from the branchial head. A remnant of the velum inherited by all of the higher vertebrates is the hyoid mechanism—an important anatomical landmark.

According to the current explanation (Gegenbaur(4) and others) of the origin and structure of many head parts and particularly of the jaw and hyoid elements of the facial skeleton, the visceral head skeleton consists of a longitudinal series of homodynamous skeletal pieces which developed as supports of the walls of the gill slits of the ancestral cartilaginous fishes.

As the gills are bilaterally symmetrical, the gill cartilages are paired, a right and left gill bar form with accessory parts, a branchial arch surrounding the pharynx. They are tied more or less closely to the ventrolateral walls of the cranium.

The branchial skeleton has been more or less modified and the anterior part (of indefinite extent) has lost its branchial functions by the disappearance of the gills. The parts that remain vary in number and form in different species.

The vertebrate jaws are assumed to derive from the first pair of permanent gill bars, the hyoid element from the second pair, and so on.

The mouth is accordingly the first pair of gill slits united into one opening. The number of gills, possessed by the ancestral type, fore and aft, is a topic still being studied. The stimulating effect of these studies on the imagination has been extraordinary.

It is our purpose to analyze the composition of the vertebrate head, beginning with the elements present in amphioxids, who have maintained themselves against the age-long assaults of the environment, are still going strong as vital facts in organic life, and seem unmindful of human assertions that they are not ancestral, not vertebrates or fish—but mere ‘weeds’ in the garden of vertebrate life; then, to study the myxinoids, whose skeleton ‘has no morphology at all’ and who also do not ‘belong’ to ‘true’ vertebrates. I am convinced that we cannot comprehend vertebrate morphology until we learn the structure of the Velata.

At present we can go no further back in the vertebrate stock than *Amphioxus*, if, as is essential, we are to study the vertebrate head in living forms and trace its history from the egg to the adult.

By starting with *Amphioxus* and continuing through the myxinoids, we are following the natural path of organic development. Starting with the sharks, already far advanced in specialization, the most important, the decisive stages in head development are never seen. By starting with *Amphioxus*, we include all living members of the vertebrate stock in an ascending series and are not led to deny anatomical relationships, or to invoke degeneracy or aberrancy or use any other term of avoidance in our comparative studies. To call *Amphioxus* a ‘weed’ which has crept into the vertebrate garden and to say that the marsipobranchs do not ‘belong’ only expose our ignorance and disinclination for hard work.

Nature knows no ‘weeds.’ The amphioxids and the marsipobranchs exist and it is our privilege to account for them with knowledge and understanding. Looking at the matter from another and correct viewpoint, we are fortunate to have

these animal types to show us the way of vertebrate development.

Amphioxus is the most primitive living organism belonging to the vertebrates. Its structure is relatively simple compared to the forms above it, yet in some of its tissues and organs it exhibits notable differentiation and specialization e.g., it has developed chondroid skeletal tissues to give firmness and resiliency to the body as a whole and to individual parts.

The branchial organ has been greatly extended in the work of food gathering by ciliate feeding—a process brought over from embryonic life and used throughout adult life. In this it retains the ancestral condition which is passed on to the larval petromyzonts, but not to the myxinoids, where the young issues from the egg as a gnathic feeder.

All vertebrates, including Amphioxus, have in common a group of ancient anatomical characters which are the fundamentals of vertebrate structure. Some of them are:

1. Cephalocaudal polarity.
2. Strict dorsoventral orientation.
3. Dorsal central nervous system.
4. Notochordal axial skeleton ventrad of the nerve cord.
5. Alimentary canal ventral of and parallel to the notochord.
6. Respiratory gills, organs perforating the anterior end of the endodermic gut.
7. Circulatory apparatus with its pumping organ ventrad of the gut.
8. A bilateral and segmented trunk muscle supplied by nerves which leave the central nerve cord at suitable intervals.
9. A specialization of the anterior end of the nerve cord—brain.
10. Organs of special sense associated with the brain.

During the long process of the differentiation and specialization of organs and parts involved in the evolution of the vertebrate phylum, up to man, these fundamental characters are changed but little, for they appear at some stage in the development of each individual and most of them are recognizably present in the adult animal. More recent characters, less ancient and of secondary structural importance, are the

ones we must rely upon to trace the genetic relations between the groups of vertebrate animals, and we here present a score or more structural characters which show the descent, with modification, of the myxinoids from their amphioxine ancestors and their structural connection to vertebrates higher in the scale of development.

1. Prebranchial head (trigeminal head).
2. Transition form of the myotome and its relation to the spinal nerve.
3. Amphioxine nerves.
4. The velar mouth.
5. The velar nerves.
6. The nasal septum and its nerves (amphioxine snout).
7. The primitive pregnathostome jaw mechanism.
8. The trigeminal complex of nerves.
9. Origin and insertion of the tentacular group of muscles in myxinoids.
10. Ciliate feeding in *Amphioxus* and *Ammocoetes*.
11. Ancestral lack of paired appendages.
12. Preservation of dorsal roots on the spinal brain.
13. Distribution of ganglion cells along the spinal nerves.
14. Location of gills caudad of velum and trigeminal territory.
15. Simple character of VII nerve in myxinoids.
16. Fourteen gills with separate openings (*Amphioxus*) (*Bdellostoma*).
17. Absence of external optic chiasm.
18. Motor and sensory nerves to each section of tentacular muscles (myxinoids).
19. Cephalolateral course of the tentacular nerves.
20. The relations of the eye to the other head structures.
21. Supernumerary tentacles in *Bdellostoma*.
22. The spinal brain and its nerves.
23. Lower jaw muscles (head muscles) already present in *Amphioxus*, greatly increased in myxinoids.
24. Ribs indicated only in the form of fibrous septa of parietal muscle, and the caudal cartilaginous transverse processes of myxinoids.
25. The cartilaginous axial skeleton in the tail of myxinoids, where, beginning with the condensation in the chorda sheath of cartilaginous neural and haemal processes, which grow around the chorda and fuse into a complete girdle and which also grow dorsad and ventrad, respectively, and fuse in the sagittal plane, there is built for the

first time in vertebrate anatomy: 1) a cartilaginous axial skeleton completely encasing the chorda, 2) neural and haemal cartilaginous tubes enclosing the dorsal spinal cord and the ventral axial blood vessels. Limited to a short section of the tail in myxinoids, this cartilage axial skeleton is developed, with variation in higher forms, the entire length of the chorda.

26. *Circulus cephalicus* (myxinoids).

27. *Transversus abdominis* muscle of *Amphioxus* and myxinoids.

28. Connecting fibers from spinal cord to notochord.

29. Jaw cartilages of myxinoids and sturgeons.

30. Jaw-operating mechanism of myxinoids and sturgeons.

31. Hypophysial plate of myxinoids and the cartilago impar of sturgeons.

The existence of these anatomical characters in the myxinoids is not a case of parallel development; they prove genetic relationship of the myxinoids to the amphioxine stock on the one hand and to the higher fishes on the other.

For many years the morphology of the vertebrate head has been dominated by the theory of the branchial origin of the jaw apparatus and other head structures. The recognition of the prebranchial head clears the way for a better understanding of the origin and development of the head parts involved.

The recognition of the prebranchial head from *Amphioxus* up through the higher vertebrates as the basis of the true morphology of the head gives us a simpler and clearer explanation of head structure than was possible when using the gill-arch theory advanced by Gegenbaur. Premandibular gills are non-existent. The posterior boundary formed by the velum, a muscular iris diaphragm developed in the walls of the original mouth, is a morphological landmark of great value. The anatomical markers set up in and on this boundary persist in every vertebrate. Such are the hyoid girdle, the ear capsule, the hyoid branch of the VII nerve, the basi-cranial skeletal bars (parachordals) and their extensions.

Likewise, the amphioxine jaw bars have taken a fundamental part in building the cranial skeleton.

The close association of skeletal structures from these two primary sources of skeletogenous tissue has furnished the

material for the fundamental pattern of vertebrate head skeleton and the infinite variety of shapes and relations of the resultant skeletal parts.

Amphioxus and the myxinoids show us very ancient forms of the head in which gills do not furnish any of the building material in the region cephalad of the oral diaphragm or velum. The prebranchial head is all of the body of Amphioxus and other vertebrates anterior to the velum and hyoid. The branchial region begins immediately caudad of the velum, which organ forms a sharp anatomical boundary between the preoral buccal cavity and the postoral gill chamber.

Some of the myxinoids preserve (*Bdellostoma dombeyi*) in adult life the number of gills (eight to fourteen) which occur in the larval Amphioxus as respiratory openings before the process of creating a food-gathering organ sets in. The velum has faded as an organ of the vertebrate head in the members of the vertebrate phylum above the Velata and only traces of it are left above the marsipobranchs. Along with loss of the velum came a telescoping ventrally of the branchial structures cephalad into the territory of the prebranchial head with a compression of the parts, which hides the boundaries of the buccal cavity from easy recognition. Accompanying these changes, the velum ceased to be the important structure it was in the amphioxine ancestors, and the sharp boundary between prebranchial and branchial territories of the gut eventually disappeared, so that above the marsipobranchs it is almost lacking as a guide in morphology to show definitely where the boundary was located, leaving the hyoid, ear, and the trigeminal group of nerves as the most reliable boundary markers. The attachment of gill-like structures (pseudobranchs) to the hyoidean skeleton is a secondary acquirement, possibly due to the telescoping of the branchial region into the prebranchial territory.

The development of Amphioxus from the egg is a straightforward continuous series of transformations which last several months. However, at the end of eight hours of growth from the fertilized egg the animal bursts out of the vitelline

membrane as a minute free-swimming embryo covered with motile cilia, to live at the surface of the shallow sea. It grows rapidly in length and does this by feeding on the contained egg yolk for the first thirty-six hours of its life, then the mouth breaks through the body wall and opens a passage from the outside sea into the gut. This momentous event introduces active feeding by the larva on the microscopic organisms living in the surrounding water and marks the end of the embryonic period of growth. The animal is now a larva and the progressive structural upbuilding of the animal continues to be from head toward the tail.

During the first eight hours the egg has become, successively, blastula and gastrula, and is soon converted into an embryo of the vertebrate type of organization possessing ciliate ectoderm for body covering and locomotion and a gut cavity lined with endoderm. The mesoderm is organized and forms the body skeleton, muscles, blood vessels, and other structures. While these changes are taking place a rod of cells between the dorsal nerve cord and the food canal forms the axial skeleton—the notochord. The body now has a distinct dorsoventral orientation and cephalocaudal polarity. Thus, left and right sides, dorsal and ventral surfaces, and anterior and posterior ends of the body come in as important morphological characters. Compare this with the blastula stage—nearly a sphere!

A day and a half is the length of time required to convert the egg of *Amphioxus* into a free-swimming, fish-shaped animal with a well-organized head, trunk, tail and tail fin, twenty-two pairs of muscle segments supplied by spinal nerves from the spinal cord. With external openings of the body, the mouth and anus, first gill slit, and anterior neuropore, which opens into the brain enlargement of the spinal cord. The neuropore is the earliest stage of the nose.

To complete the organization and maturation of the structures which form the body of *Amphioxus* to their final form (not size) requires about six months' more time.



The building up of the lancetfish out of the fertilized egg goes forward before the observer in an orderly fashion without delay for about ninety days, when the growth processes slow down, the animal settles to the bottom and enters the sand, where it passes through a period of adolescence, gradually maturing until it attains adult form and structure and a considerable increase in size.

The growth of *Amphioxus* from the egg is conveniently divided into four periods:

<i>Stage</i>	<i>Duration</i>
1. Embryo—from cleavage to perforation of the mouth, at which time the trunk muscles begin to function, replacing ciliate locomotion, but cilia persist till later .....	36 hours
2. Larva—from perforation of mouth to completion of eight (fourteen) pairs of gills and about fourteen myotomes which mark the critical stage .....	3 months
3. Adolescence—from eight (fourteen) gills to the completion of the organs of the body. A period of active growth and maturing ..	3 months
4. Young <i>Amphioxus</i> —having acquired the essentials of adult organization, it grows in size to adult volume .....	

The mouth first appears as a "disc-shaped thickening of the ectoderm" (Willey(7)) ventrad of the first myotome and near the midventral line of the body. As it grows it fuses with the endoderm, and the center of the disc so formed breaks down. This hole in the wall of the head is the mouth.

Soon the front part of the wall surrounding the mouth swings inward about the posterior border as a hinge, carrying the mouth deep into the head beneath the chorda, so that the mouth, from its original longitudinal position at the surface of the head, is now placed transverse to the long axis of the body and forms a partition across the body separating the prebranchial head from the branchial region. No gills are laid down in front of the mouth, which is now the velum. The gills begin just behind the velum. The chamber (buccal cavity) left in front of the velum by the invagination of the mouth is partly enclosed by the downgrowth of skin folds on the left side of the head, leaving a long slit—the buccal aperture, opening out in the median ventral line in front of the velum. Along the free borders of the lips of the buccal

cavity, finger-like projections of the skin are pushed out by small cartilages developed in the lips. These projections become the buccal cirri and the cartilages within them join up serially to form the jaw bars of the adult animal.

The buccal cavity is lined by the ectoderm derived from the outer surface of the head. This ectodermic layer covers the anterior face of the velum, while its posterior face is covered by the endoderm derived from the branchial gut.

As the tissues of the body differentiate the velum becomes a complex structure firmly tied to the body wall.

As soon as the mouth has been put in the velar position (or during the process of its translation thereto) the jaw bars make their appearance: first, as a small bud on the right lip or rim of the buccal chamber. Followed by other buds forming a row on the edge of the lip, these separate units of the cartilaginous skeleton of the jaw bars join together in the form of a long rod lying in the substance of the lip, while the surface projections grow out as the buccal cirri. Before the right jaw bar is complete, tentacular buds are formed near the base of the left lip, which at this time extends nearly vertically as the posterior border of the buccal opening, which opens out on the left face of the head, but soon slides down to its median ventral position. The velum has already taken up its position as a vertical curtain in front of the gut.

The muscles of the ventral body wall from the region of velar attachment grow forward into both lips of the buccal opening and furnish the inner and outer groups of muscle which move the jaw bars and buccal cirri. They are innervated by the third to seventh dorsal spinal nerves of the head.

This short sketch of the growth of *Amphioxus* from the egg is intended to picture the arrival of the mouth (velum), buccal cavity, and jaw bars in their positions in the developing head, as parts of a growing animal busily engaged in building up an amphioxine individual by reproducing in succession ancestral structures most of which remain in adult life, some of which, however, fade away before maturity as no longer required.

Ancestors of *Amphioxus* have been pen-pictured by several zoölogists. Ancestors in the flesh have not yet been found. For actual study we are limited to the embryos and adults of the few known species. No matter what the ancestral history of these structures has been, in the living, growing lancetfish, they are clear-cut organs of the present-day amphioxids, and if the nearest vertebrates above them are genetically related to amphioxids, there should be evidence of it in the structure of other Velata (myxinoids).

Some problems connected with the development of *Amphioxus*, such as larval asymmetry, need not be taken up here, as they do not affect the structural outcome in essentials and the irregularities in growth of some parts are no greater, if as great, as occur in vertebrates higher in the scale.

What is the morphological value of the structural facts gained from a study of *Amphioxus* from the egg to adult condition? Shall we accept the factual evidence of structure as we find it or assume that we have overlooked important structures or that they did not show in the process of construction, and then fill the assumed gaps in our knowledge by an active use of the imagination? Let us take the case of gills. In no period of the life of *Amphioxus* are gills formed in front of the hole in the head wall which is converted into the enduring mouth of the animal. The same is true of the myxinoids and other velates and higher vertebrates. Nevertheless, vertebrate morphology is heavily loaded with the conclusions resulting from the assumed existence of an indefinite number of prevelar gills, for no better reason than that the theory of the origin of the vertebrate mandibles and other head parts from cartilage gill bars seemed to demand it.

Of particular interest is the formation of the mouth and related parts, its translation and final relations to the head as a whole.

Starting with the assumption that the mouth of vertebrates is derived from a pair of gill slits, some students advance the view that this opening in *Amphioxus* is equal to one-half of the mouth of higher forms, i.e., to the gill slit of one side

of the body only—viz., that of the left side. How much simpler to take this hole in the head wall for what it really is—the ancient opening into the food canal. This hole is far older than gill slits; it lies cephalad of all gills. Furthermore, *Amphioxus* forms no gill slits in front of this perforation, yet preoral gill slits are assumed to have been present in varying number in the ancestors of *Amphioxus*. In the myxinoids M. Furbringer(3) thought there had been two or three anatomically distinct mouths formed in the course of their development, but I cannot find any facts to support the assumption.

In order to arise from the gill bars the mandible and hyoid would have to develop caudad of the velum. The anatomy and embryology of the Velata show that the mandible (Meckel's cartilage) of higher vertebrates is the basal part of the transformed amphioxine jaw bars, which arises in front of the velum, and that the hyoid element of the head skeleton is furnished by the skeletal condensation formed in the walls of the velum, a structure always prebranchial in position.

Not every cartilaginous condensation in the facial skeleton of the head is a gill cartilage.

Not every pocket or pit formed by the lining membrane of the food canal is a branchial pocket and not any of the pre-velar depressions are true branchial structures.

Not every contact of internal wall of the food canal with the external head covering is an incipient gill slit.

Among the ancestral characters which *Amphioxus* has transmitted to the myxinoids are ancestral relations of the dorsal spinal nerves, the fibers connecting spinal cord and notochord, and the transverse abdominal muscle.

On account of these and other structural identities, there is a proved genetic relationship of the amphioxids to the marsipobranchs which to ignore is to refuse to follow along the path the vertebrate stock has made its way from the simpler to the more complex, from the lower to the higher conditions of organization. Following this natural path, we

find the processes of organization displayed as well as the end results.

In the lower vertebrates the emphasis is not on gill arches, gill bars, or other skeletal elements of branchial organs. That stage of structure had not yet arrived and the morphology of the prebranchial head within which the jaws were formed was established before they were developed. This fact is basic. Another fact of equal importance is the primitive separation of the anterior part of the food canal into a prebranchial section (stomodaeum) lined with ectoderm and a branchial chamber formed at the front end of the digestive gut (mesenteron) and lined with entoderm, both of which take part in the formation of the head. This division was early marked by an adjustable partition, the velum, built between the two.

A third fact of morphologic importance is the early formation of the hyoid skeleton to support the velum.

This hyoid support was laid down where the velar partition joined the body wall. The chondroid skeletal bar formed here is the oldest piece of specialized head skeleton, except the chorda. It is not derived from the tissues or structures of the branchial region. Its occasional relation to respiratory structures in higher forms is secondary.

Once established, this oldest skeletal piece, except the notochord, has remained as a permanent boundary monument, a fixed landmark in head anatomy. Like other and later skeletal pieces, it has been subject to transformation in shape, to change in function, but it has held to its original anatomical position as regards its connection with the cranium. Its distal end has been swung forward and backward, inward and outward, as required by the mechanics of its use, in different vertebrates, as part of the head skeleton.

In higher forms it has been subject to a variety of segmentations and attachments. When jaws were formed, it served in some forms to suspend them from the cranium, since it, and not the jaws, had at that time firm attachment to the cranium above.

The vertebrate velum is thus, from the standpoint of comparative morphology, a very important organ. Only the Velata among the living members of the vertebrate stock possess it in adult life.

Its occurrence in embryonic and larval life in other vertebrates deserves further study. Huxley(5) described the velum of the larval frog.

The velum introduced into head anatomy the hyoid, which separates the trigeminal head from the vagal or branchial head.

For these and other reasons the Velata form a division of the vertebrate stock, comprising all vertebrate animals, having a velum (in the adult) which separates the prebranchial head from the branchial chamber. Most of the velate vertebrates are extinct.

Phylum: Chordata

Subphylum: Vertebrata

Division A: Velata

Division B: non-velate vertebrates

All living forms are water dwellers of world-wide distribution and often locally abundant. The amphioxids and myxinioids are wholly marine; the petromyzonts, partly marine and partly fresh-water forms.

The velum separates the vertebrate stock into two great groups, the ancient Velata and the more recent non-velate forms. In the myxinioids we doubtless have one of the last stages in the phylogeny of the velum.

The velum arose as a regulating valve for the control of the inflow of the respiratory and food-bearing water into the buccal cavity and the pharynx. It consists of an infolding of the wall of the gut at the hind end of the prebranchial cavity (buccal cavity, stomodaeum) supported by a chondroid or hyaline cartilage skeleton, the oldest skeleton of the head except the chorda.

It arose while vertebrates were small animals with the habit of ciliate feeding, which does not require the movement of the body as a whole. Ciliate feeding depends almost entirely

on the activity of the ciliate cells of the buccal cavity and pharynx (*Amphioxus*) or it is tied up with, and strengthened by, the pumping activity of the 'branchial' muscles (*Ammocoetes*). In the latter case we have the first cause of the decline and disappearance of this method of food capture. In ciliate feeding minute and microscopic forms of life, suspended in the water, are drawn into the food canal by the action of the ciliate cells lining it.

The velum, like other organs of the body, varies in form and structural details in different members of the group, but is less modified than many other organs whose comparative morphology have been fully established, e.g., vertebrate kidney, ear, etc.

Its most perfect and its most primitive form yet found is shown by *Amphioxus*, where it has the shape and action of an iris diaphragm and is vertical in position.

The development of the velum in *Amphioxus* is instructive, both ontogenetically and phylogenetically. Its development shows that the velum arises as the primitive mouth and remains throughout life the mouth.

In *Ammocoetes* the amphioxine disk-shaped velum is split vertically by a sagittal incision into two lateral valves or curtains which incline forward into the buccal cavity, so that the velum slants from above down and back at an angle of about 30°. The free inner edges of the curtains meet in the middle line of the body to close off the buccal from the branchial cavity and are pulled apart when a free passage of respiratory and food water is needed. During metamorphosis it is much changed structurally and its position in *Petromyzon* is nearly vertical.

The velum and the later developing jaw apparatus are tied to the axial skeleton by the velar ring, beginning in *Amphioxus* and becoming more definite and complicated as we ascend the scale.

The branchial pumping mechanism of *Ammocoetes* is far more powerful than the ciliate cell colony of the buccal cavity of *Amphioxus* for pulling a stream of water into the food

canal and in the larval lamprey has almost entirely replaced cilia in this work, which had grown too heavy for them to adequately perform. While the branchial water pump could increase its capacity beyond all requirements of the body, the extension of ciliate activity was limited by the geometric law of increase of volume of a current of water flowing through a tube. Doubling the diameter of the tube increases the capacity four times. Besides this, the cilia pull only on the surface of the solid rod of water in the buccal cavity, while the pull of the branchial pump is exerted equally over the whole cross-section of the column of water. The branchial pump draws in more water per unit of energy expended and is thus a more efficient mechanical device.

In the myxinoids the disk-shaped amphioxine velum is cut half through in the midventral line and the halves moved right and left up onto the sides of the pharynx wall, thus forming the broad velar valve plate characteristic of these forms, which springs dorsolaterally from the auditory and hyoid region of the skull and extends back into the pharynx nearly horizontally as a double-walled plate valve supported internally by a skeletal frame of cartilaginous bars, springs, and cross pieces all hinged to the hyoid by the main suspensory cartilage.

The velum in myxinoids offers little resistance to swallowing large pieces of food and is adjusted to their raptorial and gnathic feeding.

In all the Velata the velum is supported by an internal skeleton and its functions assisted by an external velar skeleton. In all cases the internal velar skeleton is composed of chondroid bars, which lie between the free folds of the velum, and is carried by the hyoid ring attached to the body wall.

In *Amphioxus* the external velar skeleton coincides with the hyoid ring, which is formed: 1) by the distal parts of the tendons of attachment of the velum to the chorda in the mid-dorsal line, 2) to the sheet of connective tissue forming both the wall of the body cavity and internal aponeurosis of the



myotomes from the chorda down to their ventral border and from here, 3) to the midventral line by the aponeurosis of the ventral visceral muscle, which attaches to the ventral border of the myotomes. Outside the visceral muscles a plate of chondroid tissue (in the metapleural folds) supports and renders elastic the walls of the buccal cavity.

The conditions which brought about the decline and disappearance of the velum included the steadily increasing body size, which called for the capture of more food than ciliate feeding could supply, and the transformation of the proximal ends of the amphioxine jaw bars into a prehensile mandibular mechanism for the capture, holding, crushing, and tearing of larger food animals.

The mandibular jaws are the reaction to physiological necessity accompanying the change of food habit from ciliate feeding to raptorial or gnathic feeding.

The transformation of the amphioxine jaw bars into the myxinoid protrusible jaw is less complicated and more direct than is that of, for instance, branchial cartilage into ear bones in the mammals.

The stages in the formation and fixation of the primary Meckel's (mandibular) cartilage from *Amphioxus* to the ganoids and of the palatoquadrate (maxillary) cartilages from the myxinoids to the ganoids show how the parts of the mechanism were produced.

#### SKELETON

Usually only the chorda and cartilage parts are referred to as skeleton in the literature of the myxinoids. Cartilage, however, forms only a minor part of the skeleton, though its most conspicuous and specialized part.

The following considerations will aid in the comprehension of the nature and structural relations of the cartilaginous skeletal frame of *Bdellostoma* to the other organs and tissues of the body and also as a brief for the origin and development of the skeleton in the vertebrate phylum.

1. The whole ancestral fibrous connective-tissue skeletal frame should be kept in view. This fibrous connective-tissue frame keeps all of the organs of the body in their places and is only assisted, not supplanted, by the chondroid condensations in limited areas. The cartilage parts appear, as necessity arises, by the conversion of a part of the fibrous skeleton into chondroid connective tissue of several kinds, the organism using here one, there another kind of cartilage.

2. There is continuous formation and growth of chondroid tissue throughout the life of the individual. The cartilage parts are completed stage by stage, but never finished.

The whole skeleton increases and extends itself with increase in size of the body—large individuals have chondroid deposits not present in smaller animals.

3. All cartilage parts subject to flexion are thin enough to bend without rupture, since the amount of cartilage is small, compared to the fibrous tissue inclosing it, these cartilages apparently serving to restore by their elasticity the shape held by the part at rest, e.g., the mandibular, velar, tentacular, and nasal cartilages.

4. Cartilage parts normally subject to little or no flexion, or called upon to resist pressure, are thicker and heavier and serve as supports and enclosures, e.g., parachordal, base-plate, and ear-capsule cartilages.

The continuous growth of the cartilage (or bone) skeleton is less pronounced in higher forms and usually ends when the 'adult' condition is reached.

In *Bdellostoma* new cartilage is added when and where it is needed. The lines of stress remain much the same with increase in age, but with increase of mass, resulting in stronger muscular pulls and other stresses, portions of the skeleton which were sufficient for younger conditions become inadequate and cartilage is formed in places where no cartilage was present before, e.g., upper jaw, caudal fin, hypophysial plate, posterior prolongation of parachordal deposits in chordal sheath, cartilage in ligament from lateral labial to trabecular horn. For other examples see the list below (figs. 1, 2, 12, 15, 17).

For a long time the relations of the skeleton of the velate vertebrates to fishes above them remained nebulous. It was the thorough study of the primitive skeleton of the members of this group that disclosed the essential nature of skeletal structure and the phylogeny of the process of building a definitely localized supporting skeleton of, 1) fibrous (fig. 9), 2) chondroid, and, 3) osseous elements out of all-enveloping and penetrating connective-tissue framework of the body.

The skeleton thus derived does not supplant the primitive connective-tissue frame of the body—it merely reinforces it. It is only the localized condensations of harder and harder materials in the ancient skeletal frame—localized hardenings to guard and support the softer parts and to absorb and localize the increasing stresses and shocks which the connective-tissue frame could not stand without rupture or distortion, but which were, in the smaller ancestral animals, well within the limits of the mechanical capacity of fibrous connective tissue.

J. Müller's work on the myxinoids is accurately descriptive of their structure as far as he went. His attempt to relate their structure to that of higher forms is summed up in his conclusion that their head structure is largely unlike that of higher forms. His conclusions were shaped by his philosophy of animal structure and growth and they influenced Gegenbaur to omit the vertebrates below the elasmobranchs in forming his visceral-arch theory of the origin of many head structures. This theory has dominated vertebrate head morphology since the early seventies. Leaving out of consideration some extravagant extensions of this theory, it seemed to be applicable to the structure of living vertebrates from the elasmobranchs on. Its application to the lower vertebrates has delayed accurate knowledge of these forms. The theory does not fit the facts.

J. Müller(6) was puzzled by the arrangement of the cartilages forming the head skeleton of myxinoids and for the most part gave up the attempt to homologize them with the head skeleton of the fishes above them. Such skeletal parts

are "nicht in der Plan der übrigen Wirbelthiere gehörinde Bildung"; "auser dem Plan der Wirbelthiere," he concluded.

It is plain that neither Müller nor Cole saw the skeleton of the myxinoids as a functioning mechanism. They sought only to homologize the cartilage pieces of myxinoids with the cartilages of fishes higher in the scale, like the sharks, where a 'plan' of organization had been set up.

After long intensive study of the cartilaginous skeleton in Myxine, F. J. Cole(2) records his conclusions as follows:

In fact, I am disposed to believe that much of the myxinoid skeleton is recent and sesamoidal (as indicated by Pollard) and therefore has no morphology at all! . . . and may represent neomorphs . . . that distinguishing feature of the mature myxinoid skeleton—the fusion of the parts into one continuous whole . . . [also] as far as Myxine is concerned, there is not a single independent cartilage in the entire skull, except a few of the nasal rings and the cartilage of the fourth tentacle.

The following list of independent cartilages shows that besides the cartilages mentioned by Cole, there are many others in the skull and that they occur especially well developed in the caudal region as well, where the process of extension and fusion of originally independent cartilages can be followed through to the completion of the enclosure of the chorda, spinal cord, and axial blood vessels in a continuous cartilaginous investment composed of a cartilage sheath about the chorda with its neural and haemal arches and spines. Here in the tail of *Bdellostoma* the cartilage skeleton has formed the parts usually found in the axial skeleton of higher vertebrates. In the myxinoids the development of the axial caudal cartilages has gone farther than in the head region behind the parachordals.

Extra (i.e., independent) cartilages forming part of the skeleton of *Bdellostoma* situate in the denser fibrous connective tissue strands of ligamentous bands and sheets. They are small bars, plates, or islands of hyaline cartilage.

1. Free nasal rings.
2. Fourth tentacle.

3. Free cartilage nodules on both faces of fourth tentacle.
4. In velar suspensory ligament.
5. Edge of 'quadrate' at hyovelar plate.
6. In anterior margin of maxilla (dental pad).
7. In median nasal fold, near dorsal end.
8. In skeletogenous sheath of chorda—cephalic end.
9. In skeletogenous sheath of chorda—caudal end.
10. In sheath of spinal cord—caudal end.
11. In fibrous nasal capsule, between the cartilage bars.
12. In pseudocartilage pad near base of lateral labial cartilage.
13. In ligament connecting cornual and lateral labial cartilages.
14. Free dorsal-fin cartilages.
15. Free ventral-fin cartilages.
16. Cloacal thread gland support.
17. Anal valve plate, and others.

The statement that the myxinoid skeleton is sesamoidal, and therefore 'has no morphology at all,' seems to indicate that Cole(2), like Johannes Müller(6), had a mental picture of vertebrate morphology which all vertebrates must conform to (more or less), else they are without form and do not belong to the morphologically elect.

But the Velata are ancient forms of vertebrates; they have perpetuated their stock through the ages and some of them still flourish in the seas of the globe. They are less specialized than any other group of vertebrates and present a series of stages in the development of the skeleton up to the formation of hyaline cartilage.

The hyaline cartilage skeleton of the vertebrate head arose early in the life of the phylum between the amphioxid and the myxinoid stage of development. The hyaline cartilage skeleton of the myxinoids of to-day appears independently in seven distinct regions of the body, five of them in the prebranchial head. In these seven provinces hyaline cartilage is called forth in the fibrous skeleton by urgent and persistent physiological needs. The cartilage of most of the provinces ultimately become more or less connected, but the cartilages of the caudal and branchial provinces remain throughout life unconnected with any other.

These provinces are: 1) velar; 2) mandibular; 3) maxillary; 4) nasal; 5) hypophysial; 6) branchial; 7) caudal.

Amphioxus and the marsipobranchs are ancestral in many ways; their structure and manner of growth show that they do 'belong.' Our failure to comprehend their genetic relationship is due to our lack of knowledge of the structures more recent vertebrate forms have changed or omitted, of the old structures they have discarded and of the new structures they have developed in the process of transforming structure to meet physiological needs.

In the rush and hustle of the egg to become a full-grown organism in the shortest possible time, much structural detail has been omitted and ancestral morphology is now sketched only in outline in the growth of the individual.

'Sesamoidal' or not, the method of cartilaginous skeleton building that we find in myxinoids is the result of efforts of the organism to meet the necessities of life. It is the *ancestral vertebrate method*. Still used by the adult myxinoids, it is largely, though not entirely, confined to embryonic stages of higher vertebrates, where stages of development lasting throughout life in myxinoids are rushed through or omitted in order that a more or less definite skeleton built according to 'plan' may be had as soon as possible.

A good example of skeleton formation in *Bdellostoma* is the independent cartilage formed in the diffuse ligament connecting and tying together the dorsal edges of the lateral labial and the trabecular cornual cartilages near the origin of the cartilage of the third tentacle from the lateral labial (fig. 1). The first is an ancient cartilage inherited from amphioxine ancestors, the second a much later development, a forward extension of the trabecula. The ligament is composed of fibrous connective tissue in which elastic fibers are numerous and is a thickened band (without sharp boundaries) of the general covering aponeurosis of this part of the head. It is continuous with the aponeurosis of the muscle group palato-ethmoidalis profundus.

In the center of the ligament, usually nearer the lateral labial cartilage, is found a small cartilage of varying shape and size composed of one or more (up to twelve) colonies of

# ABBREVIATIONS FOR ALL FIGURES

<i>A</i> , arm artery	<i>ll</i> , lateral labial (amphioxine jaw bar)
<i>a</i> , chorda tip	<i>M</i> , perichondrium
<i>ar</i> , articular facet	<i>Mx</i> , maxilla
<i>ap</i> , autopalatine	<i>m</i> , myotomes, muscle
<i>B</i> , blade	<i>mn</i> , mesial motor nerve
<i>b</i> , separate chondrification of chorda tip	<i>mx</i> , maxillary nerve
<i>C</i> , cartilage	<i>N</i> , nasal chamber and folds
<i>cc</i> , trabecular horn	<i>n</i> , nasal flap and bar
<i>c</i> , posterior end of cartilage of chorda tip	<i>O</i> , ophthalmic nerve
<i>ch</i> , coracohyoideus branch of hypobranchial nerve complex	<i>oc</i> , occipitospinal nerve
<i>ci</i> , cartilago impar; <i>g</i> , hypophysial plate	<i>P</i> , pharynx
<i>D</i> , dorsal tooth	<i>Pt</i> , pterygoid
<i>d</i> , parachordal shoulder of chorda	<i>p</i> , anterior palatine nerve
<i>dp</i> , dermopalatine	<i>pa</i> , mouth papilla
<i>E</i> , fenestra of ear capsule	<i>pd</i> , dorsal layer of palato-ethmoideo-profundus muscle group
<i>e</i> , constrictions of chorda sheath	<i>pm</i> , middle layer of palato-ethmoideo-profundus muscle group
<i>F</i> , chorda	<i>pv</i> , ventral layer of palato-ethmoideo-profundus muscle group
<i>f</i> , hypophysial valve flap	<i>Q, PQ</i> , palatoquadrates
<i>fo</i> , foramen between parts of base plate	<i>R</i> , right
<i>fx</i> , fiber joint	<i>S</i> , ethmoid
<i>G</i> , ganglion	<i>Sy</i> , symplectic
<i>g</i> , hypophysial plate in wall of nasal canal	<i>s</i> , symphysis
<i>H</i> , brain (fig. 7) hyoid	<i>s'</i> , posterior position of ethmoid horns
<i>Hm</i> , hyomandibula	<i>sp</i> , sphenolateral
<i>Hy</i> , hyoid	<i>T 1 to 4</i> , tentacles
<i>h</i> , hypophysis	<i>T</i> , trabecula
<i>ht</i> , heart	<i>t</i> , tendon or ligament
<i>I</i> , cartilage island; in figure 15, fenestra 1	<i>u</i> , upper lip
<i>ih</i> , interhyal	<i>V</i> , blood vessel
<i>J</i> , lower jaw	<i>v</i> , velar valve end
<i>Ju</i> , jugale	<i>w</i> , buccal cavity
<i>K</i> , trabecular commissure	<i>Y</i> , outline of anterior border trabecular commissure
<i>L</i> , cartilage columella	<i>1 to 6</i> , segments of base plate
<i>l</i> , lower tip	<i>I to V</i> , gill arches and gills

## Muscles, in figures and text

<i>am</i> , adductor mandibulae	<i>hm</i> , hyomandibularis
<i>ce</i> , copulo-ethmoidalis	<i>pep</i> , palato-ethmoidalis profundus
<i>cp</i> , copulopalatinus	<i>pes</i> , palato-ethmoidalis superficialis
<i>cg</i> , copuloglossus s. and p.	<i>pg</i> , palatoglossus
<i>cor</i> , coronarius muscle	<i>rhy</i> , retractor hyoideus
<i>cqs</i> , copuloquadratus superficialis	<i>rmd</i> , retractor mandibulae
<i>cqp</i> , copuloquadratus profundus	<i>te</i> , tentaculo-ethmoidalis
<i>heg</i> , hyocopuloglossus	<i>to</i> , transversus oris
<i>hcp</i> , hyocopulopalatinus	

cartilage cells varying in number from one cell to hundreds of cells. Usually there is one large cartilage ( $\frac{1}{2}$  to 1 mm. long) with other islands of cells grouped about. Some of the small islands are only one cell thick; others, two or more cells thick, all lying within the fibrous band.

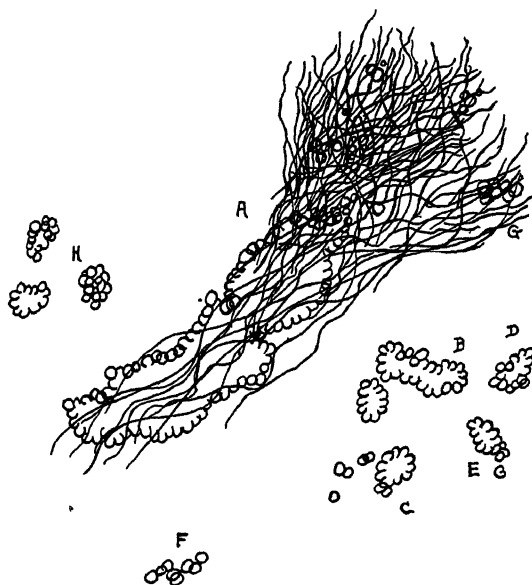


Fig. 1 *Bdellostoma*. Surface view of cartilage in the ligament connecting the trabecular horn to the lateral labial. The growth of the cartilage is along the thickened strand of fibers and usually begins nearer the lateral labial cartilage, which may send out a thin blade of cartilage into the strand. A, the first cartilage to form; B to H, later chondrifications. Ultimately all these will be united by lateral growth. The fibers of the ligament are indicated only in the A part of the figure and the cartilage cell groups only in outline.

The larger groups are distinctly yellow or brownish in color; the smaller groups and single cells, colorless in unstained preparations mounted in glycerine or in balsam.

Another example is the entirely distinct, sharply defined fibrous ligament connecting the posterior nasal bar with the hypophysial bar and trabecula at their junction in fenestra 1 or the trigeminal foramen. In *Bdellostoma* this ligament usually remains fibrous, in *Myxine* it chondrifies by the deposition of cartilage cells which unite the cartilages named.



Regarding the genesis of cartilage in *Bdellostoma*, and the several types of chondral tissue that have been classified by Cole and others in the marsipobranchs, they form a graded series from prochondral fibrous connective tissue to the so-called hard cartilage, a form of hyaline cartilage. I can confirm most of these observations for *Bdellostoma*.

The relatively simple chondromucoid tissue abundant in *Ammocoetes* I find in *Bdellostoma* in the velar valve, applied for the most part to the inner face of the mucosa in a sheet of varying thickness and continuity, where it serves to maintain the form of the valve plates, which they would otherwise not do on account of the large lymph spaces included between them which are intermittently flooded and emptied. If this kind of chondroid tissue has been found in *Bdellostoma* or myxinoids by others, I have not seen the reference. It is practically identical with the chondromucoid so abundant in the head of *Ammocoetes*. With this addition to the list, all the kinds of chondral tissues known for marsipobranchs occur in *Bdellostoma*. In figure 2 are shown stages in the transformation of fibers into cartilage cells taken from the haemal bands in the tail region of the larva before hatching, which in *Amphioxus* and the myxinoids has as the primitive or ancestral 'skeleton' already acquired extensive differentiation to perform its functions.

Recognizing the continuous fibrous skeleton, it is to be expected that the next efforts of the organism to strengthen this skeleton, now strained to capacity by ever-increasing mass and activity, would be by local deposits of stronger, more resistant material, and from *Amphioxus* on, we find chondroid tissue continuously evolved within the ancestral connective-tissue skeleton by the transformation of some of the cells into the new type of skeletal material. When cartilage no longer sufficed to meet the stresses of ever-increasing mass and activity, a third type of connective-tissue skeletal material was evolved and we find bone produced within the skeletal frame in vertebrates above the myxinoids and cartilaginous fishes. The deposition of lime salts is a chemical func-

tion of osseous tissues, just as chondrification is a chemical function of chondroid tissues. Most living vertebrates use all three skeletogenous materials. The myxinoids belong to the chondral stage, not even experimenting with calcareous incrustations of any of their cartilages. Their connective-

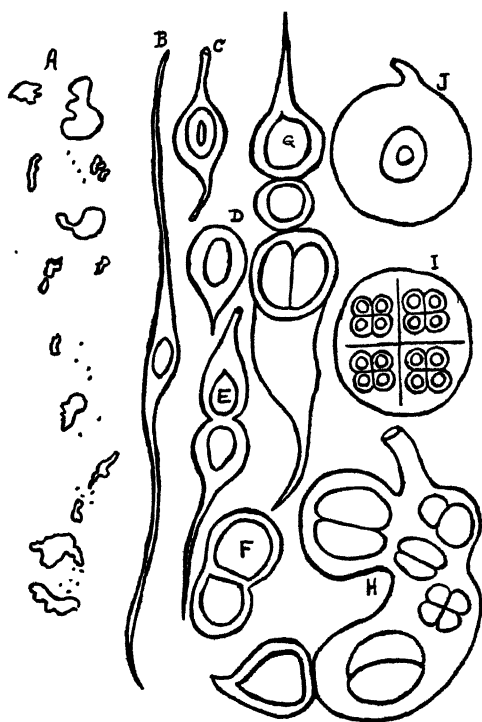


Fig. 2 *Bdellostoma*. A. Cartilage islands behind the parachordal, from a part of the haemal border of the skeletogenous sheath of the chorda of a larval *Bdellostoma* (before hatching). In the adult they have grown together into a continuous strip of cartilage. B to J, seven stages illustrating the growth of cartilage cells from fiber cells of the skeletogenous layer of the chorda.

tissue chemical reaction produces chondrin, mucin, and gelatin, although they have developed horn for capping their teeth. We can only conclude that the remarkably continuous skeleton of Myxinoids is the *normal* result of the growth of chondral skeleton in the ancestral fibrous skeleton in animals of their size and ancestral history rather than an abnormal

or unnatural structural outcome and that the skeleton of higher forms is inherited directly from the myxinoids. Now the ancestral continuous skeleton is broken into pieces, new parts added, old parts omitted until each group of vertebrates has formed a head skeleton suited (more or less) to its physiological needs, built according to its 'plan,' but always on the ancient morphological foundation stones of the amphioxine chorda, velum, and jaw bars. The breaking up of the cartilage skeleton in higher forms is consequently a secondary manifestation and the 'continuous' cartilage skeleton fades out of vertebrate anatomy. Joint formation, however, has begun even in the myxinoids, e.g., the base plate, where the thick, hard, and stiff segments of brown cartilage are flexibly joined together with fibrous hinges, and the fourth tentacle, which has been separated from the lateral labial cartilage, is now joined to it by strong tendons. In the petromyzonts the process is carried farther in the fiber and membrane joints between many of the cartilages of the head. In *Ammocoetes* there is not even the minimum amount of jointing present in myxinoids. In both myxinoids and *Ammocoetes* the hyaline cartilage skeleton is laid down as a number of separate pieces which continue to grow along the lines of stress until they become more or less continuous.

The separation of the skeleton into pieces and parts by means of highly organized joints, found in higher forms—e.g., mammals—is a differentiation connected with the need for flexibility between parts grown inflexible by increased mass and hard materials.

Cartilage cells arise in the fibrous connective tissue in the places they will occupy. I have found no evidence of migration other than the extension of cell groups by the expansion of growth which brings about the fusion of originally independent cell colonies. Examples of extension and fusion of cartilage islands are found in *Bdellostoma*:

1. In the chordal sheath posterior to the parachordals.
2. In the chordal sheath from the tip of the tail to the cloaca.
3. In the ligament connecting lateral labial to trabecular horn.

4. In the caudal end of the sheath of the spinal cord.
5. In the lateral borders of the hypophysial plate.
6. In the haemal bands along the ventrolateral borders of the chorda.
7. In the bands of the dorsal wall of the nasal capsule.
8. In the cover plates of third and fourth fenestrae of the skull.
9. In the ligament from posterior nasal bar to hypophysial bar and trabecula and others (figs. 1, 2, 3, 4, 6, 7, 11).

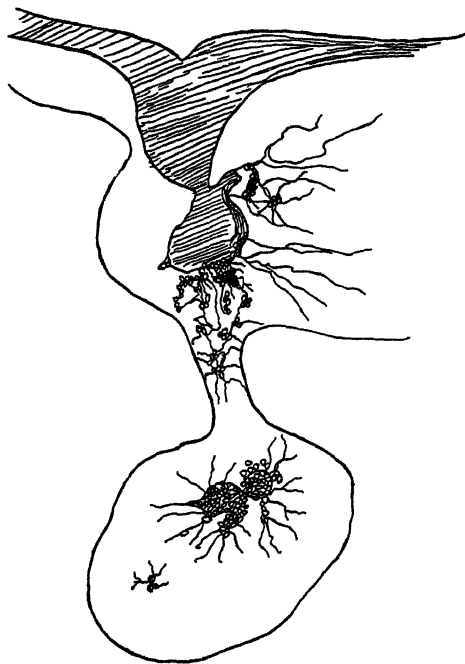


Fig. 3 *Bdellostoma*. Sagittal section of cartilage wall of nasal capsule and median nasal fold. The solid cartilage of the capsule wall, shaded. The groups of cartilage cells growing in the axis of the nasal fold are suspended in the fibrous network, filling the cavity of the fold. Only a part of the fiber structure is shown.

Myxinoid morphology is definite enough and it is illuminating because it is ancestral, phylogenetic morphology.

The structural chaos of the head skeleton of myxinoids was in the heads of the morphologists, not in the heads of the myxinoids. The difficulty was due to the attempt on the part of the anatomists to make myxinoids conform to the struc-

ture, e.g., of elasmobranchs, and this could not be done in accord with preconceived ideas of what vertebrate anatomy should be. Hence anatomists knowing the 'plan' of creation could only conclude that the myxinoids do not 'belong.'

Cartilage skeleton began in bands, cords, tendons, sheets, and masses of connective-tissue skeleton which became further developed as skeletal supports to take up the manifold stresses

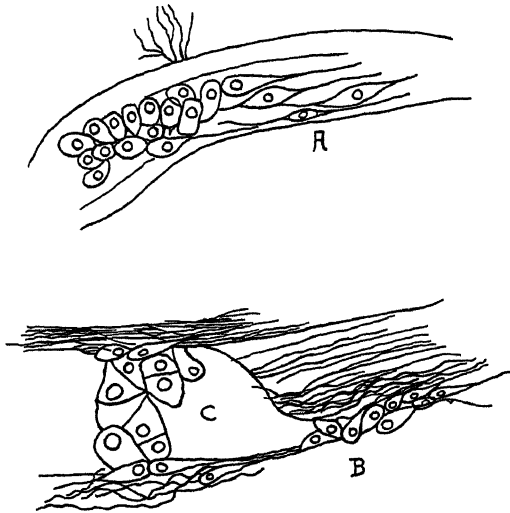


Fig. 4 *Bdellostoma*. A. Sagittal section of gill cartilage from larva before hatching, to show the conversion of connective-tissue fiber cells into cartilage cells. B. Section of ear capsule, showing the growth of cartilage at the surface by the transformation of fiber cells into cartilage cells. Above the letter B is a group of cells in late stage of conversion. To the left of this is the solid cartilage (C) of the capsule with a part of the cells drawn in. The white space is filled with cells like those in band of cells drawn in.

developed by the muscles and external forces. This type of membranous skeleton is shown in *Amphioxus*.

As shown by comparative anatomy and embryology, a denser form, a more resistant tissue than one composed of white and yellow fibers, even when strengthened by a ground substance, was needed and cartilage appeared, being developed out of the primitive connective-tissue cells along with the fibers. Then cells, instead of growing into stellate and

elongate forms, produced masses of cells with thick walls of dense material and remained in masses by the connecting of neighboring cells together by an intercellular matrix or cement. Cartilage was thus produced in isolated masses, plates, bands, rods, and protective coverings such as the ear and nasal capsules. Separated masses and plates grew until they fused together. In this way such a cartilaginous skeleton as we find in *Bdellostoma* was evolved, possessing great continuity and flexibility. In *Petromyzon*, where greater or at least long-continued resistance to stress was required to maintain the vacuum for holding to its prey and for blood suction, a heavier skeleton was developed in the head. As compared with higher forms one of the striking characters of the skeleton of *Bdellostoma* is its continuity—most of the cartilages which are separate and distinct in higher forms show no joints.

As vertebrates increased in size, mass, and activity, the building material, cartilage, proved inadequate to sustain the stresses of locomotion and predaceous attack. To meet the new needs a new sort of strengthening of the connective and support tissues appeared, mainly along the lines and in the areas where cartilage had previously been developed. This new skeletal material, bone, was at first superposed on the cartilage skeleton; later, replaced it and also appeared where no cartilage had previously been laid down. Bone is the result of a new and different chemical reaction of the fibrous and cartilage connective and support tissues to the mechanical stresses developed in the body and its characteristic is the precipitation of calcium salts in the intercellular matrix. Having matured the process of bone production, we find cartilage gradually and almost completely withdrawn as skeletal material, leaving bone and fibrous tissues as the main supports of the body in the higher vertebrates.

In *Bdellostoma* we find enough stages of skeleton formation to clearly show us how the cartilages of the axial and head skeleton were formed. We are never able to set limits to protoplasmic activity, because we know little of the physico-

chemical conditions of life within the organism (figs. 1, 2, 3, 4, 5, 6, 7, 11, 12, 13).

In *Amphioxus* the connective-tissue skeleton already shows several kinds of chondroid changes, all histologically primitive.

In the myxinoids, however, we find not only some of the early chondroid forms, but the highest type of cartilage the vertebrates have developed—viz., hyaline cartilage, but it is not produced in the large masses found in the cartilaginous fishes.

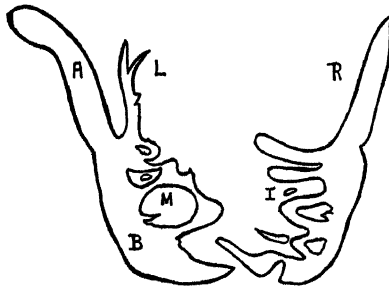


Fig. 5 *Bdellostoma*. Face view of cartilages of right and left fourth tentacle to show incompleteness of cartilage plate inclosed within the thick perichondrial tentacular plate. The arms show no lacunae and are growing at the surface. The blades of the tentacular cartilages, however, show numerous open spaces and incisions which are slowly filled in by growth at the edges.

The myxinoids are ancestral for vertebrates above them. They are far enough along in the development of the phylum to furnish definite connections with higher types, through such structures as the jaw apparatus, trigeminal complex, tail skeleton, spiracular gut pouch, etc., and since I have previously described the definite structural homology of the ancient type of dorsal nerve found in *Amphioxus* with the vanishing amphioxine nerve in myxinoids, and Schneider has found the amphioxine muscle, we are warranted in unifying the living vertebrates from *Amphioxus* to man in one genetic series.

The myxinoid is the cradle of the vertebrate cartilage skeleton; among them, so far as living forms are concerned, the cartilaginous skeleton condenses, within the substance of

the connective-tissue framework along the lines of stress, in well-defined shapes, and also in places needing protective cover.

The myxinoid upper jaw mechanism is incipient and is the earliest stage in the evolution of the vertebrate maxillary

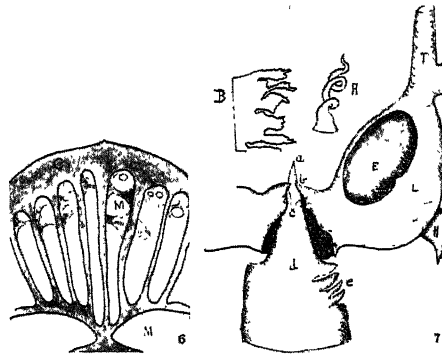


Fig. 6 *Bdellostoma*. Dorsal view of nasal capsule to show the continued deposition of cartilage in the membranous skeleton of the capsule between its primary bars. In the area of new growth there are numerous circular and oval spaces entirely free from cartilage cells.

Fig. 7 *Bdellostoma*. Dorsal view of anterior end of chorda of a young adult. All fibrous connective-tissue and cement substance removed and chorda end partly withdrawn from the parachordal socket. *a*, chorda tip covered by a separate chondrification *a-c*; *b*, anterior border of parachordal cartilage; *d*, shoulder of chorda which abuts against posterior border of parachordal saddle; *e*, indentations in side of wall of chorda produced by constricting cartilage rings—removed to show the constricting grip of the velar ring in making its attachment to the axial skeleton. The ear capsule bearing the lateral extension, the hyoid, and the anterior extension, the trabecula. *B*. Part of the incised median border of the posterior end of the ventral face of the postparachordal investment of the chorda. *A*. Illustrates one of the many variations of the chorda tip anterior to the parachordal saddle. The dotted line marks the anterior limit of the parachordal cartilage.

mechanism yet found (figs. 10, 11, 16, 17). It consists of a lens-shaped tooth-bearing body applied to the ventral face of the trabecular commissure. It closely overlies the anterior part of the lower jaw when at rest (figs. 10, 11). The anterior border of the lens curves dorsad around the anterior border of the commissure and is attached to the front border



of the commissure and to the ethmoid above it by strong fibrous sheets and bands which form the cover of the lens. This tough fibrous capsule serves as the perichondrium of the pseudocartilage and the true cartilage bodies contained in the lens. The true cartilage occurs in the form of two bilaterally placed chondrifications or when they unite in the form of a crescent. They are the beginning palatoquadrate cartilages. They lie on either side of the front border of the base of the dental papilla, i.e., in the front part of the lens.

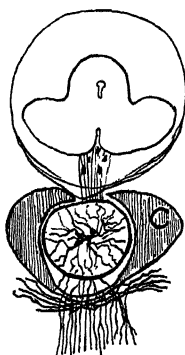


Fig. 8 *Bdellostoma*. Cross-section through parachordal cartilage, the chorda, spinal cord, to show the fibers connecting the latter to the former. The shrinkage of the parts in preparation brings the fibers clearly into view. Below the chorda are shown fibers connecting the skeletogenous layer of the chorda sheath to cartilage.

The dorsal tooth is a double structure, composed of right and left halves. It projects from the center of the ventral face of the lens, back and down into the deep V groove formed by the upfolded lower jaw plates (Meckel's cartilages). The front ends of the anterior section of the base plate, with the issuing lateral labial cartilages, lie just ventrad and laterad of the upper jaw.

The blood supply of the mechanism is from the maxillary artery, its sensory nerves from the anterior palatine. The muscles operating the mechanism are the palato-ethmoidalis profundus group innervated by the mesial motor branch of the maxillary nerve. The nerve and artery run ventrolaterad

of the trabecular bars and send their branches mesiad into the maxillary mechanism. The arterial supply of the dental papilla is specially rich. The important difference between the jaw mechanism of the myxinoids and the vertebrates above them is the absence of the articulation of the lower to the upper jaw, usually present in the jaws of higher forms. The hitching together of the two elements has not yet been developed, but all the needed parts are formed and in place, ready to be coupled into the biting jaws which we are accustomed to think are essential to normal vertebrate anatomy.

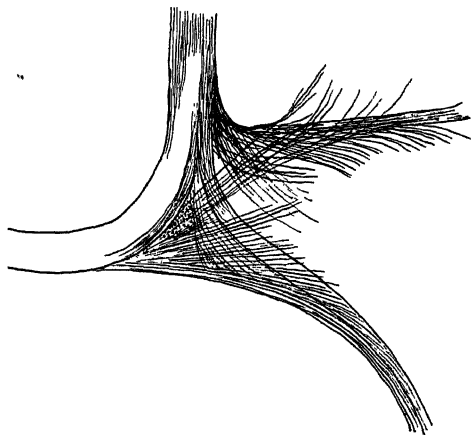


Fig. 9 Cross-section of right ventral quadrant of chorda of *Amphioxus*, showing the fiber make-up of the sheath. The continuity of the sheath fibers with the intermuscular septa and with the internal aponeurosis of the myotomes is distinct. The granular area at the angle is probably a bundle of longitudinal fibers cut across.

Having shown the presence in *Bdellostoma* of structures inherited from amphioxid ancestors, a search was made among the cartilaginous fishes for structures inherited from myxinoid ancestors. Several species from different groups were studied and we here give a list of some of the structures in *Scaphirhynchus*, an American sturgeon, of more ancient lineage than *Acipenser*, which prove beyond doubt that *Scaphirhynchus* is a transformed myxinoid which has, among other things, acquired paired appendages and has added bone to its connective tissues.

The homologies of the list are head structures such as skeletal pieces, nerves and muscles, or mechanisms such as the protrusible jaw and part of the operating mechanism of the mandible. The list is incomplete, but enough items are given to demonstrate the persistence of numerous myxinoid characters in the sturgeons, some of which have scarcely changed, while others are much modified.

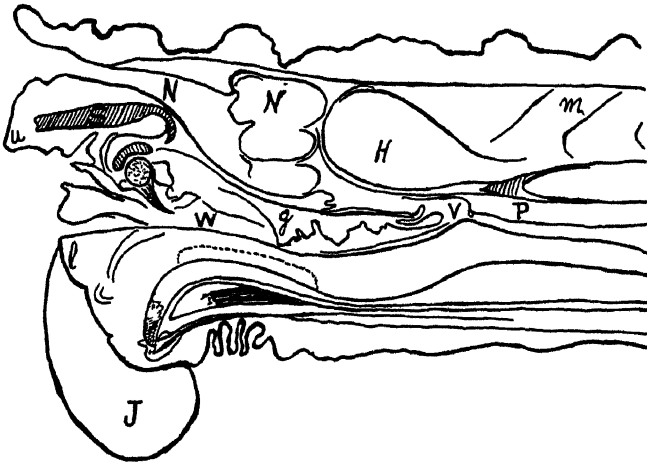


Fig. 10 *Bdellostoma*. Sagittal section of the head with protruded lower jaw. As little of the jaw shows in the median line, its internal and external positions are given in outline. At rest, it forms part of the floor of the buccal cavity; the upper jaw which forms part of the roof of the buccal cavity lies above its front border. In action it travels back and forth between the positions indicated. When fully extended, it lies in front of and below the mouth and wholly outside the head. The dorsal tooth, the dental element of the maxillary apparatus, is outlined below the section of the trabecular commissure and the long hypophysial plate is shown lying between the nasal canal and nasal chamber above and the buccal cavity below.

Comparing the two forms *Bdellostoma* and *Scaphirhynchus* as representatives of these two groups of fishes, we are able to measure the great structural advance and complication of the sturgeon over the myxinoid and to note the points of agreement and divergence and thus determine the quality and amount of proof of genetic relationship.

## Homologies

*Bdellostoma**Scaphirhynchus*

1. Protrusible lower jaw	Protrusible lower (and upper) jaws
2. Lower jaw plate	Meekel's cartilage
3. Dorsal tooth plate	Upper jaw (palatoquadrate)
4. Hyoid, vertical part	Hyomandibula
5. Base plate 1	Symplectic
6. Base plate 2	Basihyal
7. Subnasal bar, ethmoid	Median part of snout, ethmoid
8. Hypophysial plate	Cartilago impar
9. Tentacle 2	Tentacle 2
10. Tentacle 3	Tentacle 3
11. Tentacle 4	Suborbital or anterior palatine cartilage
12. Copuloquadratus profundus	Protractor hyomandibulae
13. Protractor glossus (superficial and deep muscles) and rectus	Retractor mandibulae
14. Ventral blade parietal m.	Retractor hyoideus
15. Palato-ethmoideo profundus	Adductor mandibulae
16. Anterior and posterior pharyngeal nerves	Anterior and posterior pharyngeal nerves
17. Cranial nerve VIII a	Ramus oticus

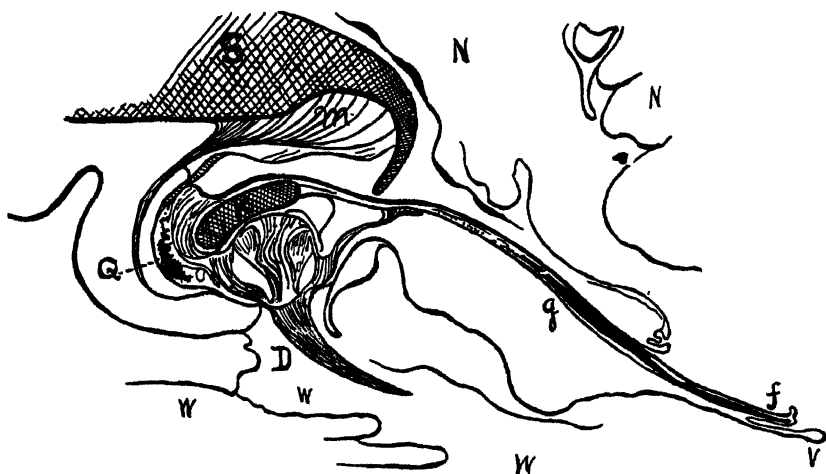


Fig. 11 *Bdellostoma*. The dorsal jaw plate and tooth from figure 10 more highly magnified. The plate and tooth are each composed of two symmetrical halves, right and left, developed in the roof of the buccal cavity cephaloventrad of the trabecular commissure. The hypophysial plate is not continuous cartilage in the median line, and where it joins the trabecular commissure it is entirely fibrous. The section of the palatoquadrate cartilage (Q) shows its relations to the front border of the maxillary body, the commissure, and the tendon of the erector muscle which has origin from the ethmoid.

The siluroids retain some myxinoid characters, but apparently not as many as the sturgeons.

Before describing the structures given in the list of homologies, explanation of the names of skeletal parts of *Bdellostoma* to be used here is in order. A detailed account will be given later.

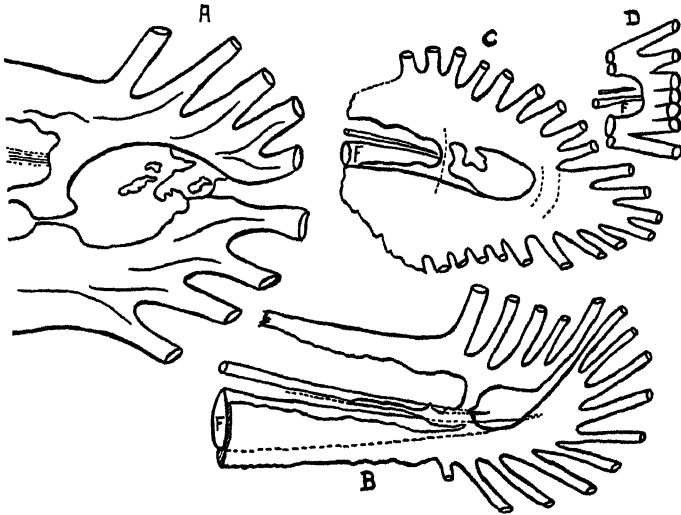


Fig. 12 *Bdellostoma*. The left sides of terminal cartilage skeleton of the tails of three individuals. Fin rays cut off. To illustrate the fusion of the neural and haemal parts. In A the anterior commissure is not quite closed and the posterior commissure still shows the line of union. In B the anterior commissure is complete, while the posterior one is open. In C both commissures are complete. The dotted lines show the attachment of caudal myotomes. In D the fusion of the fin rays shows how the posterior commissure is formed by the coalescence of the fin-ray cartilages.

1. From the central hyoid skeleton in *Bdellostoma* (and other marsipobranchs) two sets of cartilages run more or less caudad. a) The internal or intrinsic skeleton of the velum. In *Bdellostoma* they constitute the operating framework of cartilaginous rods, bars, plates, and springs used in extending and contracting the velar mouth in respiration and feeding. b) The external velar skeleton, described in the literature of the marsipobranchs as the branchial basket (Schlund Korb) which serves to keep the body walls of the

velar region (myxinoids) and of the velobranchial region (petromyzonts) expanded when not pulled upon by the muscles operating the framework of cartilage. In all the marsipobranchs, besides operating in conjunction with the velum, it serves as a water-regulating pump.

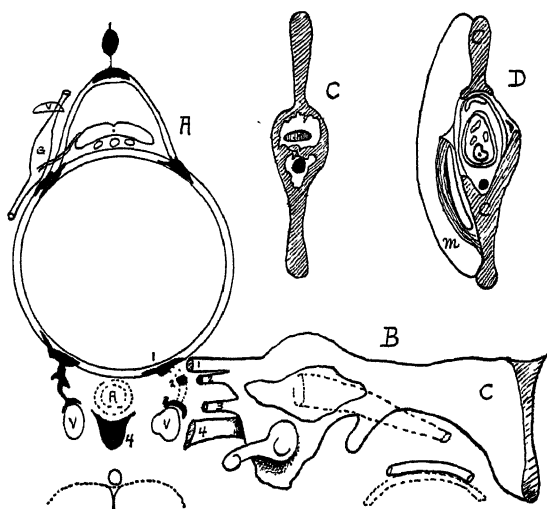


Fig. 13 *Bdellostoma*. Cross-section of chorda and cartilages of axial skeleton near anterior end of tail and side view of cartilage frame developed in this region for the support and operation of the caudal fin. B is a side view of this frame from the face of the section back into the tail fin to the anterior end of the continuous cartilage plate of the ventral blade of the fin. C. A section through the anterior commissure of tail skeleton to show enclosure of chorda and spinal cord in a continuous cartilage skeleton by the dorsal fusion of the neural processes and the ventral fusion of the haemal processes. In neighboring sections the chorda is closely invested with the cartilage. D. A section in front of the commissure to show the partial development of the neural processes. The parietal muscle of the left side is shown unshaded and the relation of the muscle cordis caudalis to the caudal lymph heart and to the tail skeleton and parietal muscle is also given.

These two sets of cartilages center in the hyoid and did not derive from branchial structures.

The hyoid is the (fig. 14) skeletal ring of the velum. It remains girdling the original location of the velum—the vertical velum of *Amphioxus*. In this position it had acquired such skeletal usefulness as a support for the prebranchial

head and branchial chamber that it was retained and further developed even after the velum had been transformed, its ventral part pushed caudad. In the caudad displacement of the ventral part of the velum—consisting in *Bdellostoma* almost entirely of transposition from the vertical to a nearly horizontal position—two other velar skeletal loops were

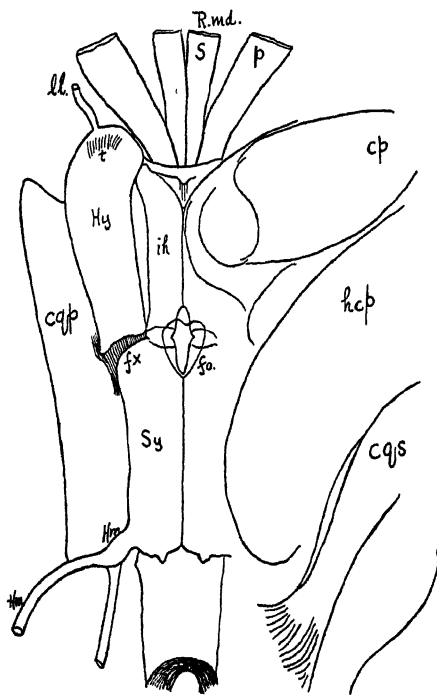


Fig. 14 *Bdellostoma*. Ventral view of base plate cartilages, showing: 1) the attachment of three superficial lateral head muscles, copulopalatinus, hyocopulopalatinus, and copuloquadratus superficialis, on the left side, and, 2) on the right side the deep lateral head muscle, the copuloquadratus profundus, which is the parent muscle of the hyomandibular protractor of the sturgeons, 3) the cut ends of the two pairs of jaw protractors which are transformed into the retractor mandibulae of the sturgeon. In *Bdellostoma* the superficial muscles *S.* are partly united with the deep muscles *p.* They are developed from a single muscle body and have the common function of pulling the ventral jaw plate and the lip skin forward and out of the buccal cavity. 4) The six (three pairs) cartilages composing the first two sections of the base plate are present in *Scaphirhynchus* as the paired hyoid, the interhyal, the symplectic, and the hyomandibula. In *Bdellostoma* the latter is not separated from the symplectic by a joint as it is in *Scaphirhynchus* and is a much smaller cartilage.

developed in the skeletogenous layer of the wall of the body. These rings have been likened to branchial arches, as they seemed to resemble such, but it is evident that these rings reaching from parachordal to hyoid base plate have never been related to the gills. They are the homologues of the cartilaginous framework surrounding the branchial region externally in *Petromyzon* and *Ammocoetes*.

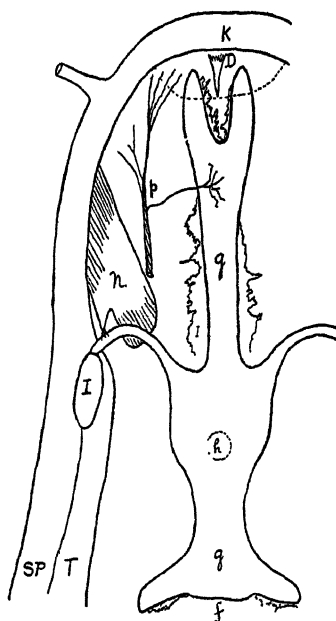


Fig. 15 *Bdellostoma*. Ventral view of the hypophyseal plate, the cartilage condensation in the fibrous plate uniting the trabecular cartilages. Above it lies the nasohypophyseal canal; below it lies the food canal (here the buccal cavity). This membrane is a continuation of the fibrous sheath of the trabeculae. A vertical line from the hypophysis gland would reach the center of the circle *h*. A part of the veloquadratus muscles take origin from the arms which curve dorsolaterally from the body of the plate to help form the foramen of the trigeminus at the junction with the trabeculae and also from the lateral borders of the body of the plate behind the arms. The broad, thin lateral plates of the nasal capsule reach ventrad in front of the hypophyseal plate, and a narrow blade of cartilage projects caudad from its posterior border to attach to the arm by a nasohypophyseal ligament, or when the blade is fully chondrified, it is fused with the hypophyseal arm near its junction with the trabecula. The anterior palatine nerve runs ventrocephalad over the arm of the hypophyseal plate to its terminal distribution in and around the maxillary apparatus.



*Parachordal cartilages (figs. 7, 8) connecting the ventral basihyoid to the chorda in the base of the skull*

The earliest chondrification is the hyoid (velar) element, from which foundation the cartilage grows and extends itself in the more primitive membranous skeleton, cephalad to form

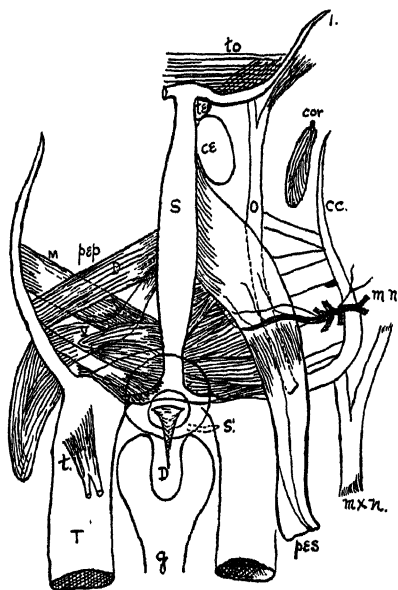
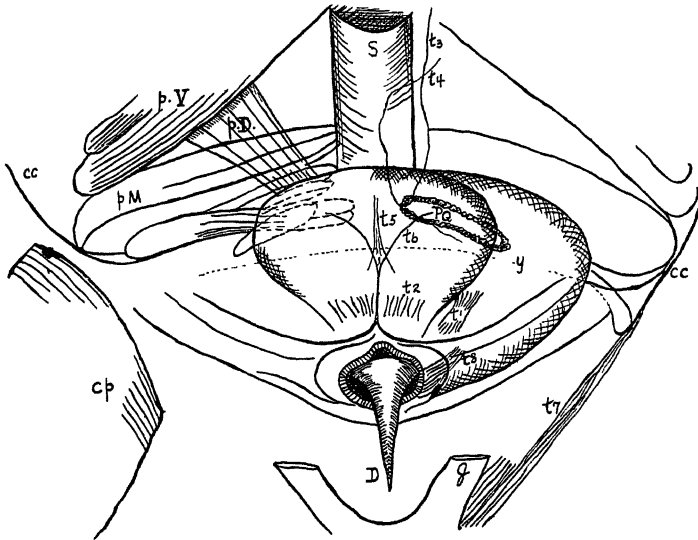


Fig. 16 *Bdellostoma*. Ventral view of palatoquadrate region, showing: a) the group of muscles which furnish the maxillomandibular muscles and their innervation by the maxillary branch of the mesial motor nerve; b) the trabecular commissure with the dorsally overlapping ethmoid with two positions of its dorsoventrally curved posterior T end; c) the anterior forks of the hypophysial plate between which the maxillary tooth is seen; d) the tendon of attachment of the palatoquadratus muscle; e) the course of the ophthalmic nerve between the superficial and deep plates of the ethmoidalis superficialis muscle. The palatoethmoidalis profundus muscles form a complex group, three layers of which are shown: *D*, *M*, and *V*, the dorsal, middle, and ventral layers, respectively.

the cranial floor, caudad and ventrocaudad with extravagant extension to form the skeleton of the velar mechanism. The anterior end of the notochord is almost completely surrounded by, and becomes fused with, this velar cartilage by the growth of the cartilage into the chorda between the elastica externa and skeletogenous layers of the chorda sheath and also into

the chorda, leading to almost complete suppression of the chordal sheath and of the tip of the chorda. Thus the velar skeleton secures a firm grip on the axial skeleton (chorda) by the soldering together of the two originally separate parts of different phylogenetic age and origin. As we ascend the vertebrate scale this process partly is repeated in ontogeny, with ever-growing reduction of the notochord. But long



Fg. 17 Bdellostoma. Ventral view of maxillary apparatus of Bdellostoma enlarged from figure 16, showing relation of the palatoquadrate cartilage to the chondroid maxillary body in a young fish. Some of its tendinous attachments (*t1* to *t8*) to the 'mucosa' are shown; *t3* is a perforated tendinous band which is united with *t4* at the crossing and with *t6*. The tendons *t1* to *t4* connect with the diffuse ligamentous band which curves ventrad about the wall of the buccal cavity, ending in the fibrous sheath of the lateral labial cartilage (the amphioxine jaw bar). Some of the maxillary bundles of the palatoethmoidalis profundus muscle are shown. The membrane of the roof of the buccal cavity is cut around the tooth base and removed. The ends of the forks of the hypophysial plate are cut off. On the right the anterior end of the lateral head muscle, copulopalatinus, ends in its attachment to the trabecula and trabecular horn. Covering the dorsal face of the lens-shaped maxillary body, between it and the cup-shaped depression in the ventral face of the trabecular commissure, is a large lymph space, and the tough fibrous sheath of the lens surrounds it and overlaps the anterior border of the commissure attaching dorsad of it to the ethmoid (figs. 10 and 11).

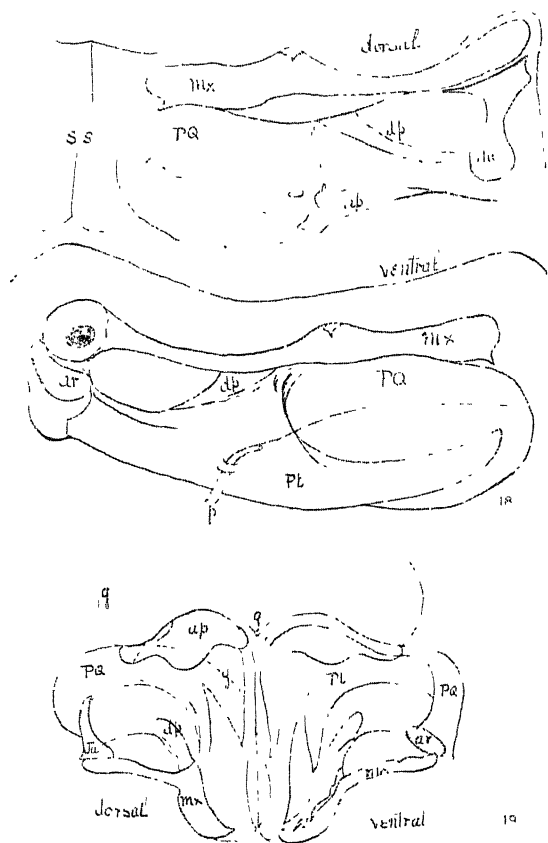


Fig. 18 *Scaphirhynchus*. Dorsal and ventral views of the right palatoquadrate cartilage and the bones as far as developed in this primitive sturgeon. This maxillary assembly of a flat plate of translucent hyaline cartilage with thickened club-shaped articular end, edged in front by a beaked bony maxilla and supported over much of its surface by a pterygoid bone, braced by bone splints at the places of greatest stress about the foramen for the adductor muscle, is an important structure phylogenetically, showing as it does how bone was introduced to strengthen the cartilaginous maxillary mechanism. The right and left palatoquadrate cartilages do not articulate in the median line; instead, a broad fibrous symphysis connects them. See figure 20 for relation to cartilago impar (hypophysial plate).

Fig. 19 *Acipenser brevirostris*. Dorsal and ventral views of right palatoquadrate cartilage and the bones belonging thereto, illustrating the production of pointed and domed upper jaw by relatively slight changes in the shape, arrangement, and direction given the plate-shaped maxillary structure found in *Scaphirhynchus*. The palatoquadrate cartilages meet in the median line and the bone maxillaries nearly so. The front edge of the unpaired cartilage (hypophysial plate) is imbricated under the posterior border of the maxillary complex and firmly tied to it by fibrous tissue.

before the acquisition of bone as a skeletal material, most of the cartilages of velar origin have disappeared without replacement. On the disappearance of the velum most of the skeleton of velar origin also disappears. The hyoid, ear capsules, and the cranial base plate (parachordals and trabeculae) remain. In highest vertebrates these also disappear, but their place and function are then taken over by bone.

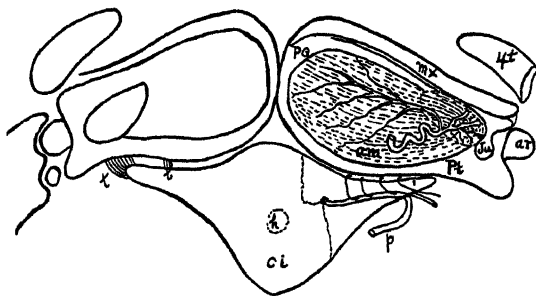


Fig. 20 *Scaphirhynchus*. Dorsal view of the maxillary apparatus and the cartilago impar to show relations. The suborbital cartilage-fourth tentacle is shown in front of the maxilla. The hypophyseal plate (*ci*), with outline of the hypophysis projected on its surface, is loosely attached to the posterior edge of the upper jaw. The anterior palatine nerve runs forward on the dorsal face of the plate, reaching its terminal on the ventral face by either perforating the plate or the membrane connecting it to the upper jaw plate. The adductor muscle of the mandible with its nerves appears in the right side and the foramen for the muscle through the palatoquadrate is shown on the left.

It is customary to assume that the basal cartilages in the midventral region behind the mouth consisting of the copular elements of the hyoid and the gill skeleton are all branchial in origin (figs. 14, 21, 22).

When the gills developed cartilaginous bar supports, they attached to cranium above at first by tendinous ligaments. No new parts were developed in cranial region for this purpose. The gills retained their fibrous attachments to skull and developed cartilaginous pieces in the course of their fibrous (supports) bars. Only later did any of these cartilages establish articulation to cartilaginous or bony elements of skull.

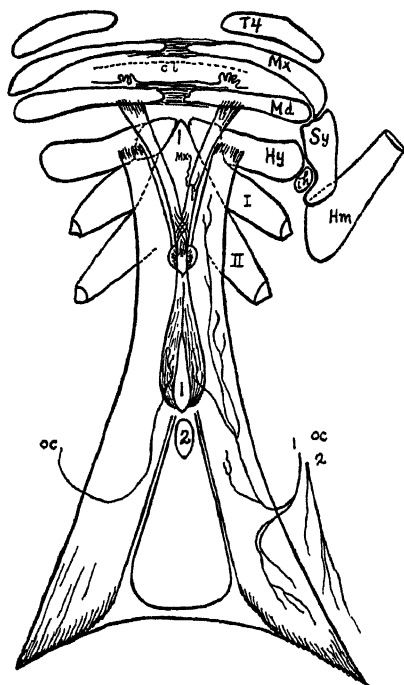


Fig. 21 *Scaphirhynchus*. Outline drawing of the ventral aspect of the jaw apparatus, hyoid, first two cartilages of the base plate, ventral ends of the first two gill bars, the toggle joint, with the retractor muscles of mandible and hyoid. The dotted line *ci* shows the position of the anterior border of the unpaired cartilage. In ventral view the jaw cartilages are seen on edge. The fibrous symphysis shows how much the upper and lower jaw cartilages fall short of reaching the median line. The two sections of the compound mandibular retractor (*r.md.*) muscles are united in the middle of their course, in front of the collar of the hyoid retractors. The anterior section derived from the protractor glossus of *Bdellostoma* is innervated by the trigeminus and the posterior section derived from the rectus of *Bdellostoma* is innervated by the occipitospinal nerve. The hyoid retractor (*r.hy.*) is the anterior part of the transformed ventral blade of the parietal muscle which in *Bdellostoma* attaches to the 'hyoid' element of the anterior end of the base plate. Compare figures 18, 19, 20. The relative proportions of the mandibular cartilage and cover bones vary in the several species of *Acipenser*. In *A. stellatus*, *schypa*, and *Guldenstadti* they are more or less confined to the angle of the jaw; the remaining distance to the midline (symphysis) is occupied by a tough tendinous prolongation of the perichondrium. In *A. huso* the cartilage is longer, but still falls short of forming a junction with its fellow at the symphysis. In the species of *Scaphirhynchus* there is a similar variation. In *S. platorhynchus* the lower jaw has a narrow fibrous symphysis, while in *Feldschenkoi*, *Hermanni*, and *Kaufmanni* the fibrous connection is much wider, with Meckel's cartilage and its dentary bone correspondingly short.

In the ventral region a cartilaginous skeleton was early established, long before cartilaginous gill supports of any size were needed, and when the gill arches, more or less cartilaginous, were developed, they utilized the median ventral skeleton for the purpose of attachment, just as they had used the ventral edges of the cranium for support.

The skeleton and other anatomical characters of the sturgeons have been studied to determine their relation to vertebrates higher in the phylum. Let us compare some of

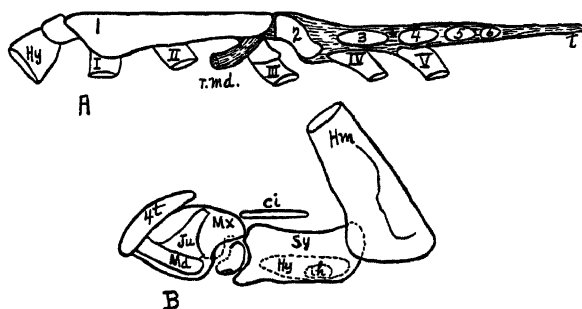


Fig. 22 *Scaphirhynchus*. A. Mesial face of right half of sagittal section of base plate composed of a longitudinal series of six median cartilages which carry the lateral elements of hyoid and branchial cartilages. The mandibular retractor has a ventrolateral origin from the posterior end of the first cartilage. The hyoid and five gill cartilages which attach to the lateral border of the base plate lie laterad of the face of the section. B. Lateral view of hyomandibula, symplectic, hyoid, and jaws to show the sequence of parts in this sturgeon. The dotted line gives the position of the interhyal and hyoid with reference to the symplectic (see also fig. 21).

the head cartilages of *Scaphirhynchus* with cartilages in the head skeleton of myxinoids, forms hitherto regarded as so much lower in the scale of organization as to be unrelated structurally.

1. The large difference between the crania of *Bdellostoma* and *Scaphirhynchus* lies in the expansion and fusion of the cartilages of the myxinoid cranium, resulting in a more or less continuous and massive piece of cartilage housing the brain and sense organs, with long and short canals leading out from the brain chamber for the passage of nerves and blood vessels.

The solid cartilaginous cranium of elasmobranchs and cartilaginous ganoids is not a primitive condition, for the cartilaginous skeleton arose as chondrification of the primitive connective-tissue skeleton, as plates, bands, tendons, etc., and in localized pieces of cartilage as in the marsipobranchs. The tendency to the fusion of the originally separate chondrifications so strikingly shown in myxinoids is less emphasized in the petromyzonts. Where flexibility was necessary, chondrification took place in relatively narrow bands or thin plates along the lines of greatest stress, e.g., external and internal velar skeleton of myxinoids. Where solidity was also needed and greater resistance required, the cartilage deposits were larger and thicker, e.g., base plate of myxinoids, facial skeleton of petromyzonts, massive crania and jaws of elasmobranchs, chimaeroids. When bone was acquired as a skeletal tissue, the massive accumulations of cartilage began to be reduced, thus saving building material, reducing body weight and size with respect to the other and more important organs which the skeleton protected and at the same time effecting better resistance to stresses, as in the bony ganoids.

The cartilago impar of *Scaphirhynchus* (figs. 10, 11, 15, 16, 17, 19, 20, 21, 22) is the homologue of the cartilaginous plate in *Bdellostoma* formed by the chondrification of the strong, horizontal skeletal membranous sheet which unites the U-shaped cartilaginous trabecular frame of the cranial floor. It is continuous with the perichondrium of the trabeculae and sphenolaterals, and since it lies ventrad of the nasohypophysial canal, it forms the ventral skeletal wall of this canal from nasal capsule back to the velum at its origin from the hyoid. It is the palatine cartilage of the prebranchial head; its ventral face is covered by the lining membrane of the buccal cavity. The cartilage is a thin plate, broadest midway of its length opposite to the foramina of the trigeminus nerves. It tapers from this oval spoon-like expansion forward into a slender bar whose forked end reaches the posterior border of the trabecular commissure. When at rest the dental armature (dorsal tooth) of the palatoquadrate lies

between these forks. The plate tapers caudad as a thin spatula whose posterior border supports the free edge of the velar valve. From opposite sides of the middle of the spoon whose concavity faces dorsad, a slender bar of cartilage curves cephalodorsolaterad to fuse with the anterior tip of the trabecula and the anterior mesial bar of the sphenolateral and thus forms a small part of the ventromesial rim of the trigeminal foramen of each side. The plate is subject to slight motion in all directions under the pull of muscles, but does not travel with the protrusible lower jaw. A large part of the veloquadratus muscles takes origin from the edges of the spoon and the posterior and lateral borders of the lateral bars.

The root bundles of the maxillary branch of the V nerve, going laterad, cross over its dorsal end in the foramen, but the anterior palatine nerves do not enter the foramen but pass cephalad along the mesial face of the ophthalmic nerve. A ventral branch of the anterior palatine runs ventromesiad across the anterior border of the bar of the hypophysial cartilage, enters the veloquadratus muscle, and later issues from the ventral part of the muscle where it is attached to the bar. The mesial part of this nerve supplies the palatoquadrate structure and its tooth. The lateral part of the nerve supplies the dorsal part of the freely movable mandibular pouch.

In *Scaphirhynchus*, as in all sturgeons, the cartilago impar has essentially the same relations to the head that it has in *Bdellostoma*. The nasohypophysial canal has disappeared and the cranial cartilages have grown in such way as to fill most of the spaces previously occupied by fibrous tissue, thus forming a cartilaginous cranium, massive and inflexible when compared to the myxinoid. In keeping with these changes, which affect the operation of the jaw apparatus, the hypophysial plate became more regular in shape by the shortening of the conspicuous projections present in the myxinoids and acquired closer relation to the now larger palatoquadrate or maxillary apparatus. It loses cartilaginous continuity with the trabecular cranial frame, is suspended from the



cranium by a forest of connective-tissue fibers, is firmly attached to the lining membrane of the buccal cavity, and moves forward and back with the protrusible jaw apparatus. Most anatomists reckon this primitive palatine plate a part of the palatoquadrate entity, which it certainly is not, as clearly proved by its origin in the myxinoids. With its long projections withdrawn the impar cartilage in *Scaphirhynchus* has a quadrangular shape, with the anterior and posterior median angles less prominent than the two lateral ones, which gives the plate a larger transverse diameter.

The lateral angles are the remnants of the lateral curved bars of *Bdellostoma* and the roots of the V nerve have the same relations in *Scaphirhynchus* as in *Bdellostoma*, particularly the anterior palatine, which runs cephaloventrad across the lateral tip of the plate to pass ventrad of its cartilage body and be distributed in the roof of the buccal cavity, the lip papillae, and about the jaw apparatus. A few of the smaller branches perforate the cartilage to reach the roof of the buccal cavity, but most of the bundles run ventrad over the anterolateral border of the cartilage or laterad of it.

In *Amphioxus* the jaw bars have little motion in the horizontal plane forward and back.

In *Bdellostoma* the lower jaw separated from the basal part of the amphioxine jaw bar has extensive travel over the basihyoid, which is quite stationary. The upper jaw remains in fixed position. Only the lower jaw is protruded.

In *Scaphirhynchus* both upper and lower jaws are protruded. The hyoid apparatus travels forward and back less than the jaws.

The toothed jaw of *Bdellostoma* is unusually movable (figs. 10, 11, 14). When at rest it lies, in its retracted position, well within the buccal cavity, its posterior end lying in a pouch formed by the folded mucosa just in front of the entrance to the velar mouth; here it is held by the club muscle. From this position it is protruded by the pull of the copuloglossus superficialis and copuloglossus profundus muscles cephalad over the anterior end of the base plate, until its posterior

end is entirely out of the mouth and the teeth, instead of pointing caudad, now point forward and upward. With such great variation in position due to functional movements, it is of interest to locate, if possible, its original position. The innervation and blood supply show that the mandible was developed at the anterior end of the buccal cavity. The nerve supply is from the trigeminal nerve by way of three branches, the third (posterior) division of the maxillary trunk, the dentalis and the mandibular nerves.

The caudad course of the posterior branch of the third branch of the maxillary nerve to the snout which innervates the region of the fourth tentacle proves the derivation of the mandibular plate from the basal section of the amphioxine jaw bars, for this nerve innervates the anterior portion of the mandibular plate and travels back and forth, in and out of the buccal cavity along with the mandible.

In *Bdellostoma* the powerful retractor muscle of the lower jaw pulls it back into the buccal cavity. Nerve supply is by the main branch of the mandibular nerve. Apparently it is not present in the sturgeons. The myxinoid protractors have taken over its function by being transformed into retractors, causing the disappearance of the myxinoid retractor. This is a good example of 'transformation' and 'change of function'—in this case effected by changing the relative position of the parts. In *Bdellostoma*, as stated above, the protractors of the mandible work around a pulley at the anterior end of the fixed hyoid (base plate). They pull the mandible forward out of the buccal cavity. In *Scaphirhynchus* the hyoid has become freely movable and relatively smaller. The mandible has been united to the maxillary element and lies in front of the anterior border of the hyoid. The mandible no longer travels back and forth over the dorsal surface of the hyoid, but remains flexibly fixed by ligament to its anterior edge. The protractors still remain attached to the mandible, but pass caudad on the ventral face of the base plate, as their ventral parts did in *Bdellostoma*, to their original origin on the ventral face of the base plate. When they contract, they

pull the mandible caudad instead of cephalad as they do in *Bdellostoma*, due to the intervention of the 'pulley' causing change of direction of the pull (figs. 20, 21, 22).

The protractor muscle of the jaw apparatus in *Scaphirhynchus* belongs to the group of lateral muscles of the pre-branchial head and in *Bdellostoma* is called *copuloquadratus profundus*. Innervation is by large motor nerve from the trigeminus in both forms (fig. 14).

In *Scaphirhynchus* the jaw apparatus lies below and loosely fastened to the ventral surface of the massive cartilaginous cranium, which is reinforced by strips and plates of bone. The ventral surface of the cartilaginous cranium curves sharply down in front of the jaws, just behind the eyes, and forms a thickened base for the snout. The jaw apparatus thus lies in a subcranial space inclosed on sides and ventral face by the skin. The ventral surface of the head is flat with no external indication of the cranial curve, because the skin forms a plane surface broken only by the lips surrounding the buccal opening and the ventral tentacles of the snout. The eyes lie in sockets partly formed by the cranial cartilage and partly by membrane. Ventrocaudad of the eyes the socket wall is membranous. Loosely attached to it, below each eye, are the almond-shaped suborbital cartilages, also called the anterior palatine cartilages. This cartilage is the homologue of the cartilage of the fourth tentacle of *Bdellostoma* and is anchored by strong tendons to the cranium. They have a thick fibrous cover and are so placed that they form part of the more or less vertical wall in front of the jaws against which the maxillary plates slide as the jaws are shoved forward, down and out of their subcranial chamber, and likewise as they are pulled back and up into it. They thus form spring buffer plates for the jaws.

The operating mechanism of the jaws in *Scaphirhynchus* is composed of the paired cartilages, maxilla, mandibula, hyoid, symplectic, hyomandibula, and some small elements together with the anterior cartilages of the median base plate. It is a toggle joint, freely suspended from the cranium by the

flexible joint between the dorsal end of the hyomandibula and the cranium (figs. 21, 22 B). The protractor hyomandibulae muscle is attached to the anteroventral border of the hyomandibula. The contraction of this muscle pulls the hyomandibula cephalad, pushing the symplectic and the jaws attached to its anterior end forward. The forward motion brings the maxilla against the vertical wall formed by the ventral curvature of the cranium and the fourth-tentacle cartilages, and the jaw apparatus is forced ventrad, everted out of its chamber, and projects below the ventral surface of the head as an open tube. The mouth, with its erected sensory lip papillae looking ventrad, is then in the best position for bottom feeding. The maxillary element of the jaws stands vertical in the front wall of the tube, the maxillary bone supporting the biting, crushing edge. Immediately behind and supporting the maxillary element is the hypophyseal plate. The mandibular element stands vertical in the hind wall of the tube, the bone dentale supporting the biting, crushing edge. Immediately behind and supporting the mandibular element is the hyoid (fig. 21).

In retracting the mouth tube, the motions of the jaw apparatus are in reverse, but the muscular pulls are supplied by the retractors of the mandible and the hyoid, two distinct sets of muscles which are inherited from the myxinoids. The hyoid retractor of *Scaphirhynchus* is the homologue of the ventral blade of the parietal muscle of *Bdellostoma* inserting on the anterior end of the hyoid (base plate) by a diffuse tendon and innervated by the occipitospinal nerves. In *Bdellostoma* their main function is to help fix the hyoid and to keep the buccal aperture open. These nerves are called hypobranchial in *Scaphirhynchus*.

The mandibular retractor is the transformed superficial and deep protractor muscles of the lower jaw (Meckel's cartilage) in *Bdellostoma*, which arise from the base plate, pass forward on its ventral face, curve dorsad around the pulley, forming the midpart of the anterior end of the base plate, pass caudad on its dorsal surface to attach to the ante-

rior border of Meckel's cartilage. By contracting they pull the lower jaw forward and out of the buccal cavity. They are innervated by the mandibular nerve.

Below the eye on each side of the head an almond-shaped cartilage fills out the orbital space (figs. 20, 21, 22). The cartilage is inclosed in a tough fibrous capsule which is continued into tendons holding it in place, but allowing some movement. It has been called the subocular cartilage, also the anterior palatine cartilage. It is the transformed fourth tentacle of the myxinoid head, as its relations to the jaw apparatus, the trigeminal nerve, and the buccal aperture clearly show. In *Scaphirhynchus* the cartilage is relatively large and the capsule correspondingly thin. In *A. brevirostris* the cartilage is small and the fibrous capsule makes up most of the body of the pad. In some species the cartilage may be absent, the whole pad composed of fibrous tissue containing fat. The skin of the ventral face of the head below the eye forms a flat surface. It is firmly attached and prevents the ventrad displacement of the tentacular cartilages. Behind the eye the cranium curves down to the level of the ventral surface of the head and body. The chamber thus formed is completed by the cartilages and muscles of the hyomandibular symplectic mechanism on the sides and the anterior part of the base plate below. In this subcranial chamber the buccal apparatus moves back and forth as it is pushed out and pulled back in feeding. In front of the jaws the skin of the ventral face of the head is reflected dorsad as a deep pocket, forming a double fold of pliable skin which is everted as the front wall of the jaw feeding tube. This skin fold prevents contact of jaws with the tentacular cartilage in the rest position. The tentacular pads function as friction or buffer pads against which the maxillary structure is pressed when the jaw apparatus is protruded and over which it travels on being withdrawn into the subcranial buccal chamber.

Some students of the sturgeons view them as more or less 'degenerate' as regards the condition of the jaw apparatus (figs. 18, 19, 20, 21, 22). Different, they are, in the structure

of their jaw mechanism, from other ganoids, but admirably adapted to their food habits. Some head structures are apparently inherited from very early ancestors, e.g., the myxinoids. In *Bdellostoma* the protrusible mandible together with the ventral part of the membranous walls of the buccal cavity is pulled out of the buccal cavity and then pulled back in. The mechanism has been often described. In the sturgeon the protrusible jaw mechanism—mandible and maxilla, etc., together with the associated membranous walls of the buccal cavity—is pushed out of the subcranial buccal chamber of the cartilaginous skull by a new mechanism, the parts of which are present in the myxinoids, but not arranged to operate as in the sturgeons; the jaws are then pulled back in by a modification of the ancient myxinoid mechanism which had there been developed in connection with the jaw apparatus. Some of the modifications of myxinoid structure which have produced the sturgeon are:

1. The reduction in size and shape of the large and long base plate (basihyoid) of *Bdellostoma* to the relatively smaller basihyoid of the sturgeons.

2. The small loose maxilla of *Bdellostoma* grew larger and became freely movable and being, like the mandible, embedded in the membranous wall of the buccal cavity, was movable along with the mandible, since it had suspension from the cranium only by way of the hyomandibula.

Later on, this free maxilla became loosely hitched to cranial floor—higher still, it is ossified to the cranium.

3. The continuous hyoid ring of *Bdellostoma* was separated into several pieces (four or more) which reached from the cranium above to the ventral body wall below. These separated pieces of the hyoid formed articular ends, where they joined together. In the sturgeons the dorsal piece, the hyomandibula, articulates with the cranium at its dorsal end and with the symplectic and operculum at its lower end. The symplectic articulates with the mandibula and maxilla and through the interhyal with the hyoid. The mandibula and maxilla have developed thin slender membrane bones applied

to the cartilages to form edges for the gape of the jaw. All these cartilages and bones are stoutly united by tendons and fascia and the whole apparatus is swung loosely in the peribuccal chamber under the cranium.

The hyomandibula projects from the cranium down and back, one on each side of the head; the symplectic, fastened to its ventral end, projects forward and slightly upward, one on each side of the head, and makes the connections with the jaws in front. Running from the lower third of the hyomandibula forward and upward is the strong hyomandibular muscle which fastens into a deep pit in the cartilage cranium dorsad of the eye. The hyomandibula forms with the symplectic a single toggle joint which straightens out when the symplectic-hyomandibular muscle contracts and pushes the symplectic forward against the jaws. However, the jaws cannot move forward, for they lie against the solid cartilaginous wall of the trabecular commissure in front of them and must perforce move down and out of the buccal chamber under the cranium. There they project free beyond the surface of the body as a palpating, sucking, prehensile tube with the jaw armature supporting its free border. The jaws together with the dorsal palatine cartilage and the ventral hyoid cartilage and the rugous covering on their buccal faces serve as the molar surface for the crushing mechanism to break up such food as crustacea and mollusks which form part of their food.

Pushed out by the toggle-joint mechanism, the sucking tube is withdrawn by two sets of muscles which are present in *Bdellostoma*; one set, the retractor hyoideus, is the transformed anterior ventral blades of the parietal muscles and is innervated by the hypobranchial (or occipital) nerve in both forms.

In the sturgeon the other is a compound muscle composed of a posterior and an anterior section.

4. The nerve supply of the mandibular tooth pads on the dorsal face of Meckel's cartilage is by the dentalis nerve, which leaves the maxillary trunk at the root inside foramen 1

and runs nearly vertically ventrad. This nerve also travels with the mandible in and out of the buccal cavity. The innervation of the two intrinsic mandibular muscles (*p.g.* and *h.c.g.*) is from the mandibular nerve trunk and the posterior border of Meckel's cartilage and its covering membrane is supplied by other branches of the mandibular nerve.

The hyoidean branch to the protractor muscle of the mandible issues, caudad of this, from the mandibular nerve just before it passes caudad of the hyoid.

What is called the tongue in the lower vertebrates is the lining membrane of the buccal cavity lying over the anterior end of the base plate (hyoid and basibranchial elements) and may be uplifted in a tongue fold or not. Intrinsic muscles are not developed in lower forms.

5. The muscles and mucosa of the vertebrate tongue are innervated by the transformed anterior spinal nerves belonging to the anterior myotomes of the trunk muscles, and these nerves are already in *Bdellostoma* so much changed from the typical arrangement of the trunk spinals that they are called occipital or occipitospinal nerves. They issue from the posterior end of the medulla as it connects with the spinal cord caudad of the cranium. As we ascend the scale to the higher vertebrates the transformation goes on until this group of spinal nerves is included in the skull and appears in anatomy as the XII cranial nerve, the hypoglossal, and it supplies the true tongue fold and the tongue muscles when present. The serial origin of these nerves seems to vary in the different groups, but in all cases they lie between the vagus and trunk spinals whether other occipital nerves disappeared in front of them or not.

If the traveling mandible is a tongue, it would be innervated by the occipital nerves in the marsipobranchs. It is, however, supplied by the maxillomandibular branches of the trigeminal nerve as in other vertebrates. In *Bdellostoma* the ventral prong of the parietal muscle extends forward on either side to the ventral face of the anterior end of the hyoid (base plate) and is there attached by a diffuse but strong



tendon. This muscle blade is the forerunner of the hypobranchial muscles of higher forms and is supplied by the occipital nerves. Since the traveling mandible utilizes all the dorsal face of the hyoid as a runway, the membrane covering it and lining the buccal cavity is kept quite free of attachments to this surface, and there is consequently no opportunity to form a tongue fold. In forms above the myxinoids where the mandible is placed in front of the anterior border of the hyoid and the gills follow behind it, the covering membrane becomes fixed to these skeletal pieces and a more or less distinct median tongue fold is often developed.

The large base plate with its cranial connection of *Bdellostoma* includes the hyoid, symplectic, and hyomandibula of *Scaphirhynchus* and cannot, therefore, be the Meckel's cartilage as has been suggested. In changing into the sturgeon condition the base plate ceases to be the runway for the mandible and takes on other relations to this jaw element. Its anterior part becomes the hyoid and supports the posterior edge of the mandible, which now has a permanent place just in front of the hyoid. The middle part of the base plate is converted into the symplectic and the cranial connecting horn becomes the hyomandibula. Thus all parts of the base plate still support and assist the jaw mechanism to function.

Anatomical evidence of the origin of Meckel's cartilage or lower jaw of *Bdellostoma* from the basal part of the amphioxine jaw bar is found in:

1. Its location on the dorsal face of the base plate, directly caudad and on the same level as the base of the lateral labial cartilages, which are the tentacle-bearing remains of the amphioxine jaw bars. They are thus the posterior piece of the serially homologous sections of the jaw bar.

2. The nerve supply of the anterior end of the jaw (mandible) is by the posterior bundles of the maxillary nerve trunk which supplies the fourth tentacle, the fifth tentacle (when present), the skin of the buccal aperture back to the anterior end of the tooth-bearing lower jaw, which in this position lies folded and horizontal upon the base plate. When

the lower jaw is fully retracted, these nerves supply the ventral and lateral walls of the anterior premandibular part of the buccal cavity. When the mandible is fully everted, it lies entirely outside the buccal cavity and stands vertical against the anterior border of the base plate. All of the lining membrane in front of it is pushed out ahead of the mandible and lies much folded, ventrad of the now ventral end of the lower jaw. Of course, all of these nerve branches are now in this folded skin and the ventral and ventrolateral border of the mandible. Except for the folding, they are in the original amphioxine position. The blood vessels, branches of mandibular artery, which run with the nerves are subject to the same displacement, traveling in and out of the buccal chamber with the mandible.

3. The nerves and blood vessels carried in this loose skin cover belong to the jaw bar series found in *Amphioxus* and their presence inside of the buccal chamber is an adaptation to the operation of the traveling jaw. Their original position was outside the buccal cavity cephalad of the base plate. When the jaw lies at rest within the buccal cavity, these nerves and blood vessels are the only nerves of the jaw bar series which change their course, cephalolateroventrad from the brain, by adding the caudad extension from the fourth tentacle on back into the buccal cavity. The explanation lies in the caudad translation of the mandible from its original position cephalad of the anterior end of the base plate.

The paired retractor mandibulae muscle of *Scaphirhynchus* is composed of two parts (fig. 21): an anterior piece attached to the lower jaw on each side of the symphysis and a posterior section with origin from the right and left sides of the caudal end of the anterior segment of the median row of cartilages to which the hyoid and the first three branchial arches are attached. From its origin, the posterior section runs ventrocephalad, the two muscles fusing together as they pass between the two retractors of the hyoid. After emerging on the ventral side of the hyoidean retractors, it runs horizontally to unite with the anterior section ventrad of the

hyoid. The junction of the two sections of the mandibular retractor is marked by a decussation of muscle bundles more pronounced in some individuals than in others. The two muscles forming the anterior section are fused or tied closely together at the junction, but soon diverge as they go forward to their attachment to the dentary bone. The posterior section is innervated by a branch of the hypobranchial nerve which runs cephalad mostly on the dorsal face of the hyoidean retractor. The anterior section is innervated by the branch of the mandibular nerve which supplies the ventral transverse muscles. The nerve runs caudomesiad to the mandibular retractor muscle and enters the ventral face of the muscle cephalad of the decussation.

The hyoidean retractor is present in *Bdellostoma* as the ventral blade of the parietal muscles which extends from below the eye to the anterior end of the hyoidean base plate and inserts on its ventrolateral border at the sides of the buccal aperture. The retractor mandibulae of *Scaphirhynchus* is present in *Bdellostoma* as the protractors of the mandible. The change of function of these muscles from protraction to retraction was effected by the permanent fixation of the mandible to the anterior border of the hyoid in the sturgeons and this was connected with a change in the method of feeding from the rasping of the myxinoids to the biting and crushing of the sturgeons. In the myxinoids the mandible is pulled or everted out of the buccal cavity by the protractors which have their origin on the ventral face of the base plate, their tendons go forward, bend around the pulley (formed by the groove in the middle of the anterior border of the hyoid base plate), from the ventral to the dorsal side to attach to the anterior end of the mandible, which lies, when at rest, well inside the buccal cavity.

The mandible is pulled back or retracted into the buccal cavity by the club-shaped muscle. In the everted position the mandible has the relation to the anterior border of the hyoid which is made permanent in the sturgeons. The hyoid pulley and the club-shaped muscle disappear. Thus the protractor

muscles are left as retractors, since they now lie entirely on the ventral face of the base plate and by contracting pull the mandible directly caudad. In the sturgeons the maxillary apparatus is much enlarged beyond the myxinoid condition and is articulated with the mandible, and this compound jaw is everted in feeding. In the sturgeons the whole buccal structure is moved forward and down—not merely the mandibular half as in the myxinoids. Using parts of the now differentiated base plate as levers, the buccal structure is pushed out and down by a toggle joint mainly composed of the hyomandibula, and symplectic cartilages pushing against the articular joint at each end of the jaws. The parts are retracted to the position of rest by the hyoid and mandibular retractors and the elasticity of the skin and tissues stretched in eversion.

The anterior section of the mandibular retractor in *Scaphirhynchus* consists of the modified myxinoid muscles, *copuloglossus superficialis* and *profundus*, which function as protractors of the mandibula in *Bdellostoma*. In the sturgeon they are part of the retractor mandibulae and have thus changed their function from protractors to retractors, because the hyoid has been reduced in size (shortened) and they no longer loop forward around the anterior border of the hyoid as around a pulley and then back to the mandible, but pass directly forward along the ventral face of the base plate to their attachment to the mandible. They take their origin from the anterior end of the muscle derived from the *rectus abdominis* of the myxinoid.

The two muscles of the posterior section of the mandibular retractor take origin from the lateral faces of the posterior end of the first median basal cartilage (fig. 22), run ventromesiad to unite in the middle line below the cartilage, and continue as a small cylindrical muscle cephalad through the collar formed at the anterior end of the retractor hyoideus. Up to the collar the muscles were inclosed between the two halves of the retractor hyoideus, but on passing the collar, the retractor mandibulae lies ventrad of the hyoidean retrac-

tor on the dorsal face of the ventral transverse muscles of the head. The muscle cylinder soon unites with the posterior end of the retractor mandibulae, as they do in *Bdellostoma*, by tendinous insertion or by interlacing of muscle bundles.

Thus the two sections of the mandibular retractor of the sturgeon form an operating unit and their separate origin, as shown by *Bdellostoma*, gives us the solution of their comparative anatomy and is another example of how myxinoid structure foreshadows that of higher forms.

The anterior section is innervated by the mandibular nerve, as in *Bdellostoma*, and the posterior part by occipital nerves. The anterior part of the mandibular retractors do not belong to the parietal musculature, for they are innervated by the trigeminus. The posterior part is innervated by spinal nerves, but has no connection with the parietal muscles, unless, as has been assumed, the rectus muscles were split off from the parietals in the early days of myxinoid evolution.

The rectus abdominis is not innervated by the occipito-spinal nerve in *Bdellostoma*. Counting the occipitospinal nerves as 1 and 2 of the spinal series, the rectus muscle is supplied by nerve 5, which enters the muscle near the tip, and consecutively by the following spinal nerves back to the cloaca. Nerves 3 and 4 supply the oblique and parietal muscles in front of the anterior end of the rectus. The first thread gland lies between the first two nerves (5 and 6) going to the rectus.

The rectus abdominis muscle runs forward from the trunk into the head as bilaterally placed ribbons of muscle forming part of the ventral body wall external to the transversus abdominis muscle and internal to the oblique. It grows stronger as it runs forward, and below the hyoidean base plate it has its greatest thickness and here it narrows from side to side, crosses mesiad over the protractor muscles of the mandible to enter between the median and lateral parts of the deep protractor muscle and attach by a ligament to the posterior part of the second segment of the base plate not far from the median line. That is the usual statement as to

its end. However, when the end of the muscle is examined, its fastening is found not as simple as this. In all cases the fibrous sheath of both rectus and deep protractor muscles develops stout tendinous bands on the ventral face of the muscles and between the muscles which tie these muscles together and gives a greater tendon anchorage than is had by the terminal tendon of the dorsal tip of the rectus muscle to the base plate. Sometimes no tendon of attachment reaches the base plate, the whole anchorage of the rectus muscle being its attachment to the two neighboring parts of the copuloglossus profundus. Cole also found this manner of attachment in *Myxine*.

The result is the operating fashion of the end of the rectus with deep protractor of the mandible, the rectus adding its pull to that of the protractor. This structural development in the myxinoids is carried further in the sturgeons. The myxinoid condition enables us to understand how the compound mandibular retractor of the sturgeon was evolved.

Omitting the branchial muscles, there are few head muscles in *Scaphirhynchus*. The long cone of muscle which runs from the lower two-thirds of the anterior edge of the hyomandibula to a pit in the cartilage cranium above and in front of the eye is composed of fibers of varying length and direction. The longest take origin in this pit, but most of them arise from the side wall of the cranium. As the muscle body passes over the lateral face of the roots of the trigeminus nerve a large motor nerve runs directly into it, sending branches forward and back in the muscle. This is the largest branch of the mandibular nerve and is given off from the motor root in the middle of the length of the root.

This hyomandibular muscle is the direct descendant of the copuloquadratus profundus muscle of *Bdellostoma*. As usually described, this muscle in *Bdellostoma* takes origin from the so-called quadrate at its near approach to the caudal end of the second division of the base plate forward along the 'quadrate' and sphenolateral to the trabecular commissure. From this origin the muscle bundles run cephaloventrad to

the inner border of the upper edge of the base plate from the posterior end of the second division of the base plate to the anterior tip of the first division, i.e., the whole length of the true basihyal of *Bdellostoma*, which in the sturgeons is separated into hyoid and symplectic. Its special nerve is the lateral bundle of the motor nerve which reaches its inner face of the posterior end, runs along ventrad of the maxillary trunk to the snout, sends branches forward and downward diagonally across the muscle, and is used up by the time it reaches the anterior end of the muscle. Other smaller branches from the motor trunk sent to the mandibular nerve also innervate it, and a few sensory branches from the maxillary nerve supply the vascular and connective tissues of this large muscle. The transformation of the copuloquadratus profundus muscle of *Bdellostoma* into the jaw protractor of *Scaphirhynchus* is linked up with the shortening of the sphenolaterals and the base plate and the segmentation of the hyoid, along with the suppression of that part of the muscle in front of the eye, the transfer of the original caudal end of the muscle to the hyoid (hyomandibula), so that the fibers run forward and slightly upward. The origin from the sphenolateral forward to the eye is retained.

This brief account of some of the head structures of living amphioxids, myxinoids, and sturgeons deals with vertebrate animals far apart in grade of development and probably in the time of their appearance on the globe, although their geological history is unknown.

We know that the development of animals has usually been accompanied by—has been expressed in—the change in the number and kind of organs of the body, in the structure of the organs, and in the tissues of which the organs are built. Change is usual, but some animals, some organs, and some tissues have reproduced themselves in this world of flux and change for untold generations without noticeable modification.

The jaw apparatus shows stages of change in the three kinds of animals named above, progressively from the simple

to the more complex, both in the form and relations of the parts of the jaw mechanism and in the tissues composing these parts. From the condition of the jaws in the sturgeons it is possible to follow the path of development to the highest vertebrates. At present we gain little by speculating on the ancestry of amphioxids, but we are on firm ground in giving them first place in the ancestry of vertebrates as we know them. With this ancestry established, the origin of vertebrates from advanced arthropod animals is untenable.

I hope to publish more of the observations on which these conclusions rest.

Morphological characters such as the prebranchial head, the velum, the amphioxine nerve, the transversus abdominis or amphioxine muscle, the fibers connecting spinal cord to chorda (fig. 8), and others are incontrovertible evidence of the direct descent of the marsipobranchs from forms possessing the amphioxine structure. Likewise, the presence in the cartilaginous ganoids (Teleostomi) of the protrusible jaw, connected with the two compound myxinoid muscles composing the retractor mandibulae, innervated as in *Bdellostoma* by a branch of the mandibular nerve and derivative of the spinals, together with the retractor hyoideus (transformed ventral blade of the anterior parietal) innervated as in *Bdellostoma* by the occipitospinal nerves (hypobranchial), is incontrovertible evidence of the genetic relation and descent of the cartilaginous ganoids from marsipobranch ancestors.

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# A COMPARATIVE STUDY OF THE CHROMOSOMES OF RODENTS<sup>1</sup>

JAMES CECIL CROSS

ONE TEXT FIGURE AND THREE PLATES (THIRTY-NINE FIGURES)

## AUTHOR'S ABSTRACT

A comparative study has been made of fifteen species and subspecies of rodents to determine the range in number and variation in morphology of their chromosomes. The number range is between 40 and  $86\pm$ , with an average number for known species of 52. In the family of Cricetidae there is little variation in chromosome number and a general similarity of the chromosomes. In the Muridae the range for known species is 40 to 42, but the chromosome morphology even of subspecies may be quite different. In Sciuridae a range of 48 to 62 has been found in different species. The greatest range in number is found in the Heteromyidae, 44 and  $86\pm$  chromosomes having been found in two species. It is concluded that the stem number for rodents is close to 48 and that fragmentation and fusion account for variation in numbers, and these affect the morphology of the chromosomes. The evidence also indicates that variations in morphology are due to translocations and inversions, and possibly deletions accompanied by translocations.

This study is one of a series of investigations which have been conducted at the University of Texas dealing with the chromosomes of rodents and other mammals. Proceeding from a phylogenetic point of view and limiting the work to a single order, a study has been made of as many species of rodents as could be obtained in order to determine the range in numbers and variation in morphology of their chromosomes and, if possible, to give some explanation for the changes that have occurred. A similar study has been made by Painter ('25) for the different orders of Eutheria. He proceeded from a genetic point of view, and wished to determine whether or not the chromomere associations had been stable, for such stability would have enabled geneticists to homologize the same chromosome in different orders. In this first study the evidence seemed to point to the possibility of extensive homology, but in a later paper Painter ('28) compared the chromosomes of the Norway rat and the house

<sup>1</sup>Contribution from the Department of Zoölogy, University of Texas. This work was done under the direction of Dr. T. S. Painter, to whom the writer wishes to express his obligation and appreciation.

This paper was constructed from the original thesis in zoölogy, presented to the faculty of the Graduate School of the University of Texas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

mouse, at that time considered by taxonomists to be in the same genus, and found morphological differences that he was unable to explain by a simple fusion or by a fragmentation of the chromosomes. He concluded from these observations that an extensive shifting of the chromatin material, such as would be brought about by translocations, had taken place between the chromosomes, since these two forms arose from a common stem. These findings coincide in many respects with those reported below.

The question of the behavior of chromosomes during phylogenesis has been of interest to cytologists since it was established that chromosomes are a part of the hereditary mechanism of animals and plants. As early as 1905, McClung, in his work on Orthoptera, suggested linkage, or fusion, as a method whereby a change of both number and morphology may have taken place. The later studies of Robertson ('16), Woolsey ('17), and McClung ('17) on Orthoptera, Metz and Moses ('15) on Diptera, Wilson ('23) on Hemiptera, and other cytologists on various insects, have indicated that fusion and fragmentation, as well as other processes, have been responsible for variation both in number and in morphology. Researches by botanical cytologists, who have been particularly active in this field for the past few years, indicate that these factors as well as others not commonly observed in animals have probably been instrumental in modifying the chromosome garnitures of various plants.

In plants the problem is complicated by polyploidy, hyperploidy, and hybridization. That these conditions obtain in plants has been known for a long time, but in animals they are rare and so would not have affected, to any great extent, either the chromosome numbers or chromosome morphology of mammals. The only cases of polyploidy reported in animals, i.e., occurring in nature, are the few instances where parthenogenesis is involved, as in *Artemia* (Artom, '11) and *Solenobia* (Seiler, '23). Polyploidy in animals would bring about an unbalanced condition of the sex-determining mechanism of the heterozygous sex, causing intersexes, as pointed

out by Painter ('25) and by Muller ('25), and individuals showing this condition would be unable to propagate themselves. Hybridization is known to occur between species of mammals, but in practically all cases some degree of sterility results, and in most of the known cases of interspecific hybridization the mating was brought about with domesticated or semidomesticated animals. All of the animals used in this investigation were taken from the fields and woods in the wild condition, and the probability of interspecific hybridization, at least in the more recent stocks, appears very small. Hybridization could have occurred between the subspecies of the fox squirrel, but in this case the chromosome numbers are the same.

Altogether, it appears that in rodents we have a group that is quite favorable for the study of chromosome behavior during speciation, and this fact, coupled with the great interest which mammalian chromosomes have attracted within recent years, has led to the selection of this order for intensive study.

#### ACKNOWLEDGMENTS

The animals used in this investigation were collected on the Pacific coast, in Arizona, and in Texas. The writer is indebted to Mr. E. L. Sumner, Jr., of the Museum of Vertebrate Zoölogy, University of California, at Berkeley, for collecting a number of specimens, as well as for classifying animals taken on the Pacific coast. All of the animals collected in Texas were classified by members of the U. S. Biological Survey, and the author wishes to express his appreciation for the courtesies shown by Dr. W. B. Bell, Principal Biologist, in charge, Division of Biological Investigation, and by Dr. H. H. T. Jackson, Senior Biologist, Division of Biological Investigations. The preserved material of one species, the kangaroo rat, was furnished by Miss Bessie League, of Texas Technological College, Lubbock, Texas, and the animal was classified by Dr. Walter P. Taylor, of the U. S. Biological Survey. The writer very gratefully acknowledges this assistance.

## MATERIALS AND METHODS

Fifteen different forms are included in this work, representing thirteen species (two having two subspecies each) taken from ten genera and five families of the order Rodentia. In all cases testicular tissue was used. When possible the animals were trapped and brought to the laboratory alive, where the testes were removed and the tubules preserved in Allen's modification of Bouin's fluid warmed to about 37°C. Some of the squirrels were killed in the woods and the material preserved as soon thereafter as possible. This is easily accomplished if the fluid is prepared in the laboratory (with the exception of adding the chromic acid and urea) and heated to the proper temperature and then carried to the woods in a thermos bottle. The testicular material can be obtained before the animal has completely expired, and in this way very successful preparations are obtained. Sections were cut at 7.5  $\mu$  and 10  $\mu$  and were stained in iron hematoxylin.

All drawings were made with the aid of a camera lucida, at a magnification of about 4100 diameters. Volumes of chromatin material were calculated by stepping off the total length of the chromosomes for a given drawing with a pair of dividers, estimating the average diameter of the chromosomes, and using length times the diameter squared to obtain the volume.

## OBSERVATIONS ON SPERMATOGONIA

Since our primary interest has been that of chromosome numbers and morphology, special attention has been given to spermatogonia. In a few cases, especially where high numbers are involved, spermatocytes were used for checking the counts; but in some instances dividing spermatocytes were not found, due to the season of the year when the animal was secured. In each case clear cells that are well within the plane of the section were studied. Drawings of two spermatogonia are given for each species and subspecies except for the kangaroo rat, the pocket gopher, and one sub-

species of the fox squirrel, but usually many other cells were drawn and counted and used for comparison. In cases where two spermatogonia have been drawn the chromosomes of one cell have been copied with a copying camera lucida and arranged in pairs in a descending order with respect to size. At the time of pairing the chromosomes were checked under the microscope in order to make allowance for foreshortening, but even with this check the method of pairing is only an approximate one. Whether or not the chromosomes represented by the X-Y pair at the right end of the series are really the sex chromosomes can be decided only after a study of spermatocytic divisions has been made, but an uneven pair is present. The species and subspecies are grouped below according to families as well as genera.

### *Cricetidae*

The family of native mice and rats, Cricetidae, is the best represented of the five families studied, and will be dealt with first. Four genera are included.

*Peromyscus californicus insignis*. This deer mouse is, according to Anthony ('28), one of the largest of the Peromysci. It is interesting to note that the cells are about one-third larger than those of the other three members of the genus. In fact, they are larger than those of any other species used in this work. The diploid number is forty-eight (figs. 1 and 2). As is shown in the serial alignment (fig. 20), several of the chromosomes are relatively quite long, and the decrease in size from the largest pair to the smallest is gradual. No exceptionally small, dot-like elements appear. Three pairs of V- or J-shaped chromosomes are very conspicuous, and one other, the fifth pair, may be in this category. There are no pronounced constrictions other than those usually found in V- or J-shaped chromosomes.  $2n = 48$ .

*Peromyscus boylei attwateri*. In this species (figs. 3 and 4) the chromosomes are quite similar to those of *P. californicus insignis*, with the exception that the decrease in size is not as gradual (fig. 21). Since the cells are only about two-

thirds as large as those of *P. californicus*, the chromosomes, as would be expected, are proportionally smaller. The five largest chromosome pairs are V- and J-shaped elements (fig. 21).  $2n = 48$ .

*Peromyscus maniculatus gambeli*. The spermatogonia (figs. 5 and 6) again show a diploid number of forty-eight. Four pairs of J-shaped chromosomes appear in the serial alignment (fig. 22). In general, the chromosomes are similar to those described for the two species just mentioned, with the exception that four pairs of small chromosomes have pronounced transverse constrictions. No small constricted chromosomes are found in *P. californicus* or *P. boylei*.  $2n = 48$ .

*Peromyscus maniculatus hollesteri*. Although this mouse is a subspecies to *P. maniculatus gambeli* just described, the diploid number of chromosomes (figs. 7 and 8) is fifty-two, instead of forty-eight. In both animals four pairs of V- or J-shaped chromosomes are encountered (compare figs. 22 and 23). The chromosomes of the two mice are very similar in general morphology, but some differences do occur which are apparently linked with the difference in number. The conspicuously constricted chromosomes of *P. m. gambeli* are not apparent in *P. m. hollesteri*; on the other hand, the latter has more small chromosomes than the former. This difference suggests that fragmentation may have caused the variation in number, but a discussion of this possibility will be taken up later.  $2n = 52$ .

*Sigmodon hispidus texianus*.<sup>2</sup> The Texas cotton rat has fifty-four chromosomes (figs. 9 and 10). Although some bends may appear in the larger chromosomes (fig. 24), no conspicuous V's or J's are present, and no constrictions are found.  $2n = 54$ .

*Neotoma floridana attwateri*. This species is the wood rat commonly found in the hills and cliffs around Austin. The spermatogonia are represented by figures 11 and 12. Most

<sup>2</sup> This is probably the same species which Painter ('28) mentioned as the Austin wood rat.

of the cells show a pair of V-shaped chromosomes and one pair that is more or less J-shaped (fig. 25). The majority of the chromosomes appear as curved rods, and the decrease in the size in the alignment is quite gradual.  $2n=52$ .

*Microtus townsendii*. This species was caught on San Juan Island near Friday Harbor, Washington, and is commonly called a meadow mouse. With the exception of a few constricted chromosomes, there are no marked peculiarities in morphology. All of the chromosomes appear as rods or dots. The spermatogonial drawings are shown by figures 13 and 14, and the serial alignment is given in figure 26.  $2n=50$ .

### *Muridae*

The Muridae are Old World mice and rats, of which two genera have been introduced into this country, *Mus* and *Rattus*. *Mus musculus*, the house mouse, has been studied by Cox ('26) and by Painter ('28). *Rattus norvegicus* is the Norway or barn rat reported by Painter (l.c.) and Pincus ('27). Pincus also described the chromosomes of the black rat, *Rattus rattus rattus*, in considerable detail. The animal reported here is the Alexandrine or roof rat, and is a subspecies to the black rat. This makes a complete survey of introduced mice and rats. In addition to these, Minouchi ('28) has given a report for *Mus wagneri*, an Asiatic form not yet found in America outside of laboratories.

*Rattus rattus alexandrinus*. As is shown by figures 15 and 16, the chromosome number (forty) is the same as that found by Pincus in the black rat. When a comparison is made between the serial alignments of Pincus (figs. 62 to 66) for the black rat and my own figure 27 for the roof rat, it is clear that in the former there are three pairs of large V- or J-shaped elements, while in the latter there is only one such pair. These subspecies, together with the two species of *Mus*, show the lowest number of chromosomes reported for rodents, the next lowest being that found in the Norway rat.  $2n=40$ .



*Sciuridae*

Two genera of squirrels are represented here, *Sciurus*, or the common tree squirrel, and *Glaucomys*, or flying squirrel. Two species of another genus, *Citellus* (ground squirrels and rock squirrels), were studied, but as favorable cells for counting were not found, these animals are not included among the fifteen species mentioned in an earlier part of this paper. However, they will be discussed very briefly. A subspecies for one of the tree squirrels will also be discussed.

*Sciurus carolinensis carolinensis*. The southern gray squirrel is a comparatively small animal, being about three-fourths the size of the Texas fox squirrel. The chromosomes are large and their morphology is very striking. Of the forty-eight chromosomes (figs. 28 and 29), about fifteen pairs are V- or J-shaped (fig. 32), with only a little more than half that number of relatively small rods. No very small dots are found. Two satellites are found, labeled *s* in the figures, but apparently they are not associated with synaptic mates. A tendency for the satellite to split before its associated chromosome showed any sign of splitting was occasionally observed (fig. 29), and constrictions that usually accompany V- and J-shaped chromosomes are encountered.  $2n = 48$ .

*Sciurus niger rufiventer*. The spermatogonial cells of the western fox squirrel are given in figures 30 and 31. The number is sixty-two in this species, while it is only forty-eight in *S. carolinensis*. This striking difference led to the measurement of the chromosomes of each species and the calculation of the ratios of chromatin material. The ratio is 1:1 for the two species, even though the fox squirrel has fourteen more chromosomes than the gray squirrel. The serial alignment of the fox squirrel (fig. 33) reveals only eight pairs of large V- and J-shaped elements as compared with fifteen pairs in the other. Furthermore, the fox squirrel has a number of comparatively small rods and dots, while the gray squirrel has no very small chromosomes. In addition to these differences, no satellites are found in the fox squirrel.  $2n = 62$ .

*Sciurus niger limitis*. This is a subspecies of *S. niger rufiventer*, and is called the Texas fox squirrel. Investigation showed that the chromosomes are so nearly alike in the two subspecies that only one drawing is given for *S. niger limitis* (fig. 36). No serial alignment is given.  $2n=62$ .

*Glaucomys volans volans*. The animal is the type species and subspecies of the flying squirrels. The calculated amount of chromatin is only about three-fourths that of the other squirrels, and the chromosomes are quite different morphologically (figs. 37 and 38). One or two pairs of chromosomes might be considered J-shaped, but the others are rod-like. The gradation is quite uniform (fig. 34), although considerable foreshortening was encountered. Four pairs of small chromosomes are constricted; otherwise the morphology is not unusual except that it is strikingly different from that of the other squirrels.  $2n=52$ .

*Citellus tridecemlineatus arenicola*. No very good cells were found for the thirteen-lined ground squirrel. One cell showing fifty to fifty-four chromosomes was studied. The cells appear to be smaller than those of the *Sciurus* group, but a large number of V's and J's are present.

*Citellus grammurus buckleyi*. As little success was met with in the study of this species as with *C. tridecemlineatus*, due largely to the fact that very few divisions were found in the material collected. The animal itself, the Texas rock squirrel, is larger than the thirteen-lined *Citellus*, and in many ways resembles the tree squirrels. The cells and chromosomes have a superficial resemblance to those of the fox squirrel, but no accurate counts were made.

### *Heteromyidae*

Only two species were studied from the family of pocket mice and rats, the *Heteromyidae*, and these belong to different genera.

*Perognathus fallax fallax*. This is the California pocket mouse. As shown by figures 17 and 18, the diploid number is forty-four. Seven or eight pairs of V-, J-, or U-shaped

chromosomes stand out very clearly. The others are all rod-like, and of various sizes (fig. 35), but very few extremely small or dot-like chromosomes are found.  $2n = 44$ .

*Dipodomys merriami merriami*. Only one cell from this animal, the kangaroo rat, was drawn (fig. 19), due to the difficulty of counting and determining the exact form of the small chromosomes. The number of chromosomes shown is eighty-six, but with such a high number it is obvious that some error in count is probable. However, this count is a close approximation to the exact number, as shown by many cells, and it is considered the minimum rather than the maximum number.  $2n = 86 \pm$ .

### *Geomyidae*

Only one species of this family is reported. It is a pocket gopher from northeastern Texas.

*Geomys breviceps breviceps*. Like the kangaroo rat, the gopher has an exceedingly large number of chromosomes (fig. 39), and again the number reported is taken to be only a close approximation.  $2n = 84 \pm$ .

### DISCUSSION

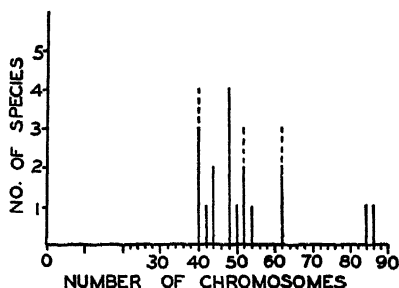
The foregoing observations show that the range of chromosome numbers for the rodent species studied extends from forty to at least eighty-four or eighty-six. Previous to this work the known range in numbers was from forty in the house mouse and black rat to sixty-four or sixty-five in the guinea-pig. Altogether, we now have data for twenty-one species and subspecies of rodents.<sup>3</sup> The results have been listed in table 1, and in text figure 1 the number distribution is given graphically. A study of text figure 1 will show that

<sup>3</sup>Lower numbers than forty have been reported by early investigators, but later work with more refined technique gives higher numbers than were found at that time. Also, Athias reported twenty-eight to thirty-four as the haploid number for *Microtus incertus*, which, if thirty-four were considered as correct, would make the diploid number about sixty-eight. For a complete list of these references the reader is referred to *Handbuch der mikroskopischen Anatomie des Menschen*, 1/2 Lebendige Masse II, S. 196-198.

TABLE 1

COMMON NAME	FAMILY	GENUS	SPECIES	SUBSPECIES	DIPLOID NUMBER	OBSERVER
White-footed mouse	Cricetidae	Peromyscus	californicus	insignis	48	
White-footed mouse	Cricetidae	Peromyscus	boylei	attwateri	48	
White-footed mouse	Cricetidae	Peromyscus	maniculatus	gambeli	48	
White-footed mouse	Cricetidae	Peromyscus	maniculatus	hollesteri	52	
Cotton rat	Cricetidae	Sigmodon	hispidus	texianus	54	
Wood rat	Cricetidae	Neotoma	floridanus	attwateri	52	
Meadow mouse	Cricetidae	Microtus	townsendii		50	
Pocket mouse	Heteromyidae	Perognathus	fallax	fallax	44	
Kangaroo rat	Heteromyidae	Dipodomys	merriami	merriami	86♀	
Roof rat	Muridae	Rattus	rattus	alexandrinus	40	
Black rat	Muridae	Rattus	rattus	rattus	40	Pineus
Norway rat	Muridae	Rattus	norvegicus		42	Painter, Pineus, Minouchi
House mouse	Muridae	Mus	musculus		40	Cox
House mouse	Muridae	Mus	wagneri	musculus	40	Minouchi
Gray squirrel	Sciuridae	Sciurus	carolinensis	carolinensis	48	
Western fox squirrel	Sciuridae	Sciurus	niger	rufiventer	62	
Texas fox squirrel	Sciuridae	Sciurus	niger	limitis	62	
Flying squirrel	Sciuridae	Glaucomys	volans	volans	52	
Rock squirrel	Sciuridae	Citellus	grammurus	buckleyi	♀	
Ground squirrel	Sciuridae	Citellus	tridecemlineatus	arenicola	♀	
Pocket gopher	Geomyidae	Geomys	breviceps	breviceps	84♀	
Guinea-pig	Caviidae	Cavia	cobaya		60-64	League
					65	Mols
Cottontail rabbit	Leporidae	Sylvilagus	floridanus		44	Painter

no typical rodent number has been found, such as we might, perhaps, expect, but on the other hand, it is clear that most species (sixteen out of twenty-one, including subspecies) fall within the limits of forty to fifty-four. The average number of twenty-one species and subspecies is 52.3; but if we exclude the kangaroo rat and pocket gopher, which have twenty chromosomes beyond the previously known range, the average for nineteen species and subspecies is 48.9, a number that is not far from the crest of the distribution curve (text fig. 1). It is clear, of course, that if many species have as high a number as the kangaroo rat, the average number for the rodents would be materially increased. On the other hand, the twenty-



Text fig. 1 A graphic representation of the occurrence of the chromosome numbers in eighteen species of rodents and three subspecies. The broken lines represent subspecies. The guinea-pig is placed at 62.

one species and subspecies studied constitute a fairly representative sample, and in view of the fact that the family Heteromyidae show species both with a very high number and with a number below the average, we should expect that further studies would not alter the average number as here given to any great extent.

In the family of native rats and mice (Cricetidae) the range in chromosome number is between forty-eight and fifty-four. There is a general similarity in the chromosome alignments, and the variation in number can be explained, in part at least, on the basis of fragmentation or fusion. Three species of *Peromyscus* each show forty-eight chromosomes, but in the two subspecies of *P. maniculatus* we find forty-eight and fifty-two chromosomes, respectively. In this instance it seems

safe to assume that forty-eight is the type number of the genus, and the deep constrictions in several of the chromosomes of *P. maniculatus gambeli*, with forty-eight chromosomes, suggest a step in the formation of the condition in *P. maniculatus hollesteri*, in which there are fifty-two chromosomes. Further evidence to support this hypothesis is that the latter subspecies has more small chromosomes than the former, and does not have the medium-sized constricted elements.

The other members of this family, the cotton rat, the wood rat, and the meadow mouse (figs. 24, 25, and 26, respectively), do not show any typical large V-shaped chromosomes such as are seen in the serial alignments of the *Peromysci*. On the other hand, in these three species the chromosomes are condensed, making it hard to identify shapes. In each of the three the irregularly curved large chromosomes might appear as typical V's or J's in a less condensed condition. The gradations of the alignments are similar, and the number differences may be explained in the same way as in the genus *Peromyscus*.

In the family of introduced mice and rats (*Muridae*) no great variation in chromosome number has been found, but the difference in chromosome morphology is very marked even in subspecies. For five species and subspecies the number range is from forty to forty-two. A careful comparison was made by Painter ('28) of the Norway rat and the house mouse, at that time placed in the same genus by systematists. This study showed that the differences must be due to an extensive shifting of chromatin between chromosomes (translocations). In his study of the black rat and Norway rat Pincus found, in addition to differences of the morphology of the sex chromosomes, some large U-shaped chromosomes in the black rat that were not present in the Norway rat. The former has forty chromosomes and the latter, forty-two. When he compared the spermatocytes found in the Norway rat and the black rat with those found by Cox in the house mouse, Pincus reached the conclusion that the spermatocytes

of the black rat and house mouse were more similar than those of the black rat and the Norway rat. In my work, the roof rat, a subspecies of the black rat, has been studied. Pincus gives at least three pairs of large V- and U-shaped chromosomes for the black rat, while only one pair of large V's is found in the roof rat (fig. 27) and one pair that is medium-sized. The difference in number of V-shaped elements indicates that probably inversion of chromosome segments has occurred within the species, or translocations, causing an atelomitic attachment of the spindle fiber.

In the squirrels (Sciuridae) the variation in both the numbers and the morphology of the chromosomes is quite marked. The numbers for the gray and fox squirrels are forty-eight and sixty-two, respectively, and that of the flying squirrel is fifty-two. The morphological relations in this family are extremely interesting. In the first place, the chromosomes of the two species of fox squirrels are very similar, in contrast to the roof rat and black rat, or in *P. maniculatus gambeli* and *hollesteri*, while on the other hand the fox and gray squirrels present a decided contrast, both as to numbers and form. The serial alignment of the fox squirrel (fig. 33) shows eight or nine pairs of V- and J-shaped elements, with about eleven pairs of small rods and dots. The seriation is closely graded. In the serial alignment of the gray squirrel (fig. 32), which has fourteen chromosomes less than the fox squirrel, fifteen or sixteen pairs of V's and J's are found, one pair of which is among the smaller elements. No really small chromosomes like those found in the fox squirrel are present. Here again the difference can be explained by fragmentation or fusion, depending on which is regarded as the type number. The calculated amount of chromatin material gives a ratio of 1:1 for these two species.

The chromosomes of the flying squirrel exhibit a morphology that would apparently set this species apart from the fox and gray squirrels. No V-shaped elements are found, and only one pair of chromosomes could be described as J-shaped. The first two chromosomes in the serial alignment (fig. 34)

are curved in shape, but they are not V's. In the same figure also appear three pairs of small constricted elements. The calculated amount of chromatin material shows a ratio of 3:4 when compared with that of the other two squirrels. When compared with the chromatin material of the *Peromyscus* group, the ratio is as 1:1. Also, the general morphology of the complex is much more like that of the *Peromysci* than that of the other squirrels. Both the superficial appearance of the cells and the analytical study of the chromosomes of the flying squirrel when contrasted with those of the fox and gray squirrels suggest the conclusion that the ancestral lineages of the two genera have been quite removed from each other. This is not the case with the fox and gray squirrels, for either a superficial or a detailed study will indicate that the two animals are close relatives, and the discrepancy between the numbers and morphology of their chromosomes is probably of relatively recent origin.

The greatest range in chromosome numbers for a single family is found in the *Heteromyidae*, although only two species of the family were studied. The pocket mouse has forty-four chromosomes, and the kangaroo rat,  $86 \pm$ . In the former there are about seven pairs of V-, J-, and U-shaped elements (fig. 35), and the chromosomes are relatively large. In fact, this species has some chromosomes as large, relatively, as any found in the rodents. The kangaroo rat has about seven pairs of V's and J's also. One or two pairs more may be present, but of this there is some doubt. A calculation of the amount of chromatin shows that the mouse has as much chromatin material in proportion to cell volume as the kangaroo rat. The dissimilarity of the chromosome constitutions of the two species is so outstanding that one doubts the validity of putting them into the same family.

The foregoing analysis of the conditions met with in rodents clearly brings out the fact that there is no general rule which can be laid down regarding the behavior of their chromosomes during speciation. An estimation of the volume of chromatin within the various groups indicates, with one



exception, that the volume of chromatin material is the same in species within a family; but in one family we may find a fair constancy in chromosome number and general morphology, while in others we have very striking differences in morphology, and a number difference of as much as forty-two chromosomes. Each family seems to be a law unto itself, and, as in the insects, we find one group deviating little in chromosome number, while in other groups the greatest diversity of number is found. These conditions naturally lead us to the question of the causes of variation in chromosome number and morphology.

Evidence has already been presented which indicates that fragmentation or fusion has been largely responsible for the changes in chromosome number, and shifting of chromatin segments, i.e., translocations and inversions, can satisfactorily explain the observed differences in morphology. A change of the point of spindle-fiber attachment is commonly due, presumably, to the inversion of the segments of a broken chromosome, although it is clear that if two rod-like elements become joined at their spindle-fiber ends, a V-shaped chromosome may be produced. In like manner, a translocation may produce a V or a J, depending upon the size of the translocated piece, if it becomes attached to the spindle-fiber end of another chromosome. These four factors may be used, theoretically, to explain the conditions as they exist in the rodents that have been studied, but they do not include all of the agencies which cytologists have suggested for explaining differences in chromosome numbers and morphology.

Among the factors which have been suggested as producing differences in the chromosome complexes in addition to those mentioned above are: polyploidy, or the addition of complete chromosome sets above the diploid number; hybridization; non-disjunction; sudden mutation, and the gradual disappearance of a chromosome.

As pointed out already, the probability that polyploidy occurs in animals is very small on account of complications that would arise due to the sex chromosomes. In the Hetero-

myidae the kangaroo rat has approximately twice as many chromosomes as the pocket mouse, but an estimation of the chromatin volumes gives a ratio of 1:1 instead of 2:1, as would be expected if the kangaroo rat were a tetraploid form of the mouse. Furthermore, the number of V- and J-shaped elements is about as large in the mouse as in the rat. These conditions indicate that polyploidy does not explain the difference in chromosome number between the two species.

Just how great a rôle hybridization has played in the evolution of rodents is difficult to estimate at the present time. Theoretically, we should expect little influence from interspecific hybridization due to the large amount of sterility that usually occurs among the hybrids of mammals.

Recent genetic and cytological studies have shown that non-disjunction is not uncommon in the maturation of animals. This condition, of course, would result in a hypoploid, as well as a hyperploid, condition in the offspring. Chromosome deficiency seems to be generally accompanied by low viability, and under natural conditions such deficient animals would not be likely to survive. The duplication of a chromosome, as in hyperploidy, does not in itself seem to handicap an animal, but the trisomic condition is an unstable one, and to obtain the tetrasomic condition necessary to insure stability would require the union of two germ cells, both of which carried extra chromosomes that were homologous. Such a coincidence seems unlikely to occur under conditions prevailing in nature, but it is a possibility, of course.

Concerning other processes which have been suggested, such as a sudden mutation involving a number of chromosomes at one time, or the gradual disappearance of a small and presumably genetically functionless chromosome, little can be said at the present time to support either of these points of view. The nearest approach to the former condition in rodents is that reported by Swezy ('28), in which the diploid numbers of forty-two and sixty-two are given for a single strain of Norway rat. No evidence at all has been found for the existence of extremely small M chromosomes in rodents.

The elimination of other factors leaves fragmentation or fusion and translocations and inversions (or deletions accompanied by translocations) as the most probable causes of changes in chromosome numbers and morphology in rodents. Whether fusion or fragmentation has played the dominant rôle in altering numbers cannot be answered. It seems probable that both processes have been operative, but neither can be conclusively established for the rodents studied. If it is assumed that fusion has been the more effective method of producing variations, then the highest numbers must be regarded as most primitive. If eighty-six is taken as the primitive number, it becomes very difficult to explain why the majority of the species show numbers that range rather uniformly around forty-eight. On the other hand, if we assume the lowest number (forty) to be the more primitive, we encounter the same difficulty of explaining why so many forms show a general range of chromosome numbers around forty-eight. The most probable explanation is, in view of our present knowledge, that the stem number was close to forty-eight and that both fragmentation and fusion have operated during speciation to change the chromosome number.

No attempt will be made to review the very extensive literature dealing with chromosomes from the phylogenetic point of view, but attention should be called to several recent comprehensive studies, with which the present work is in agreement.

In the Lepidoptera Beliajeff ('30) has studied thirty-eight species and has summarized the data of other investigators, so that altogether ninety-four species are considered. The range in the haploid chromosome number is from eleven to eighty-seven, but sixty-nine species (75 per cent) show a range between twenty-eight and thirty-one. The other (twenty-five) species are well scattered over the remainder of the range, not more than three species having the same number. In some families, as, for example, the Sphingidae, the number variation for thirteen species is small (twenty-

seven to twenty-nine), while in others it is very large, and in the family Lymantriidae it is interesting to note that the lowest as well as the highest numbers occur. *Orygia* has eleven and *Dasychira* has eighty-seven chromosomes, but the average number for all species known in this family is thirty-one. In general, species with low numbers possess relatively large chromosomes and, conversely, in species with high numbers the individual elements are small. From his comparative study Beliajeff reaches the conclusion that thirty chromosomes is approximately the original or stem haploid number for the Lepidoptera, and that deviations from this can be explained either by fusion, lowering the number, or by fragmentation bringing about a corresponding increase. He finds no evidence of polyploidy and does not believe that hybridization has played a significant rôle in the evolution of the chromosomes of this group.

It is scarcely necessary to point out the similarity of conditions in the Lepidoptera and in the Rodentia. The Sphingidae have a parallel in the Muridae of rodents, and the very wide range within the family Lymantriidae is like that observed in the Heteromyidae. The dot-like character of the haploid chromosomes of the moths and butterflies does not lend itself to a close study of chromosome form or of size, and for this reason it is not surprising that Beliajeff does not consider, as a factor in speciation, the shifting of parts of chromosomes from one element to another. As far as number variation is concerned, my own observations are in entire accord with his conclusions.

One of the most outstanding surveys which has been made on plants is the combined cytological and genetic studies of the genus *Crepis* carried out by Babcock and Navashin ('30) and their coworkers at the University of California. About one-third of the ( $200 \pm$ ) known species of this genus has been studied. Contrary to many botanical cytologists, Babcock and Navashin do not regard polyploidy or hybridization as having played extensive rôles in the evolution of the chromosomes of this group. Most of the changes observed can

be explained by relatively small intraspecific alterations of the chromosomes brought about by what is termed 'transformational processes,' by which is meant fragmentation, fusion, translocations, deletions, etc. "The transformational processes, since they could induce not only differences in chromosome morphology but also those changes in chromosome number which would account for the 6, 8, 10 portion of the series, must be considered the most basic of the processes, connected with chromosomal variation."<sup>4</sup> These general conclusions are, of course, in complete agreement with those which have been reached from a less extensive survey of the rodents.

There is a good deal of specific evidence in cytological literature both for fusion and fragmentation of chromosomes (Wilson, '25, chapter XI). Intimately bound up with the latter process is the matter of spindle-fiber attachment and the part it plays in the division of chromosomes. The x-ray work done at this laboratory has clearly shown that when the chromosomes of *Drosophila melanogaster* are broken by irradiation the fragment distal to the point of spindle-fiber attachment is lost unless it is subsequently reattached to the same or some other chromosome which carries a spindle fiber. Whatever the action of the spindle fiber may be, it appears necessary for the proper distribution of the chromosomes. In *Drosophila* the area of spindle-fiber attachment appears to be small, but many cytologists have described the attachment zone for other animals and plants as being quite broad, and in *Ascaris* the fibers have been shown to be anchored along most of the chromosome length. If the conclusions drawn from *D. melanogaster* are correct, we will have to assume that spontaneous fragmentation of a chromosome in nature would result in the loss of the piece (and ultimately of the individual because of the semilethal character of large chromosome deficiencies) unless the break took place in the fiber zone so that each fragment would have a connec-

<sup>4</sup>Babcock, E. B. Investigations in the genus *Crepis*. Carnegie Institution Year Book, no. 29, 1929-1930, page 373.

tion with the division center. It may well be that the reason some families, or larger groups, show such constancy in chromosome number is due to a restricted fiber zone, so that the chance of two broken pieces having fibers would be very small. On the other hand, if the fiber zone were a broad one, breaks could occur within it and the two pieces remain in connection with the division center. Our knowledge of spindle fibers and their action in cell division is in a very unsatisfactory state, and in view of the renewed interest it has now, from the x-ray work and from theoretical consideration, it should receive close study once more by cytologists.

There is one further point which should be emphasized and that is that the average chromosome number for rodents is about the same as the stem number assumed by Painter, when he studied species from seven orders of the Eutheria. It is surprising that in spite of the great diversity in form and chromosome constitution occasionally encountered in the Eutheria, so many species have deviated little from the presumable stem number of forty-eight chromosomes. In this connection reference should be made to the recent extensive study of Matthey ('31) on reptilian chromosomes in which he advances an ingenious scheme to explain the chromosome relationships in amphibians, reptiles, and mammals.

#### SUMMARY

1. A comparative study has been made of the spermatogonia of fifteen species and subspecies of rodents to determine the range in number and variation in morphology of the chromosomes. Six species and subspecies reported by other investigators are included in the comparison.

2. The number range extends from 40 to  $86 \pm$  chromosomes for the order, with an average of  $52 \pm$  for twenty-one species and subspecies.

3. Four genera and seven species and subspecies of the family Cricetidae show a number range of forty-eight to fifty-four, and the chromosome morphologies are very similar for all seven species and subspecies.

4. Only one species of the Muridae was studied, but the work of other investigators has brought the number to five species and subspecies. The number range is forty to forty-two, with noticeable differences in chromosome morphology.

5. For four species and subspecies of Sciuridae the range of chromosome numbers is forty-eight to sixty-two, with very striking morphological differences.

6. The number range is  $44$  to  $86 \pm$  for only two species of Heteromyidae, with a very wide difference in chromosome morphology. One species of the Geomyidae has  $84 \pm$  chromosomes with a morphology very similar to that of the species of Heteromyidae having  $86 \pm$  chromosomes.

7. Most of the differences in number may be explained by fusion and fragmentation. These factors may also affect the morphology of the chromosomes. Other factors that may have brought about morphological changes are translocations and inversions, and deletions accompanied by translocations.

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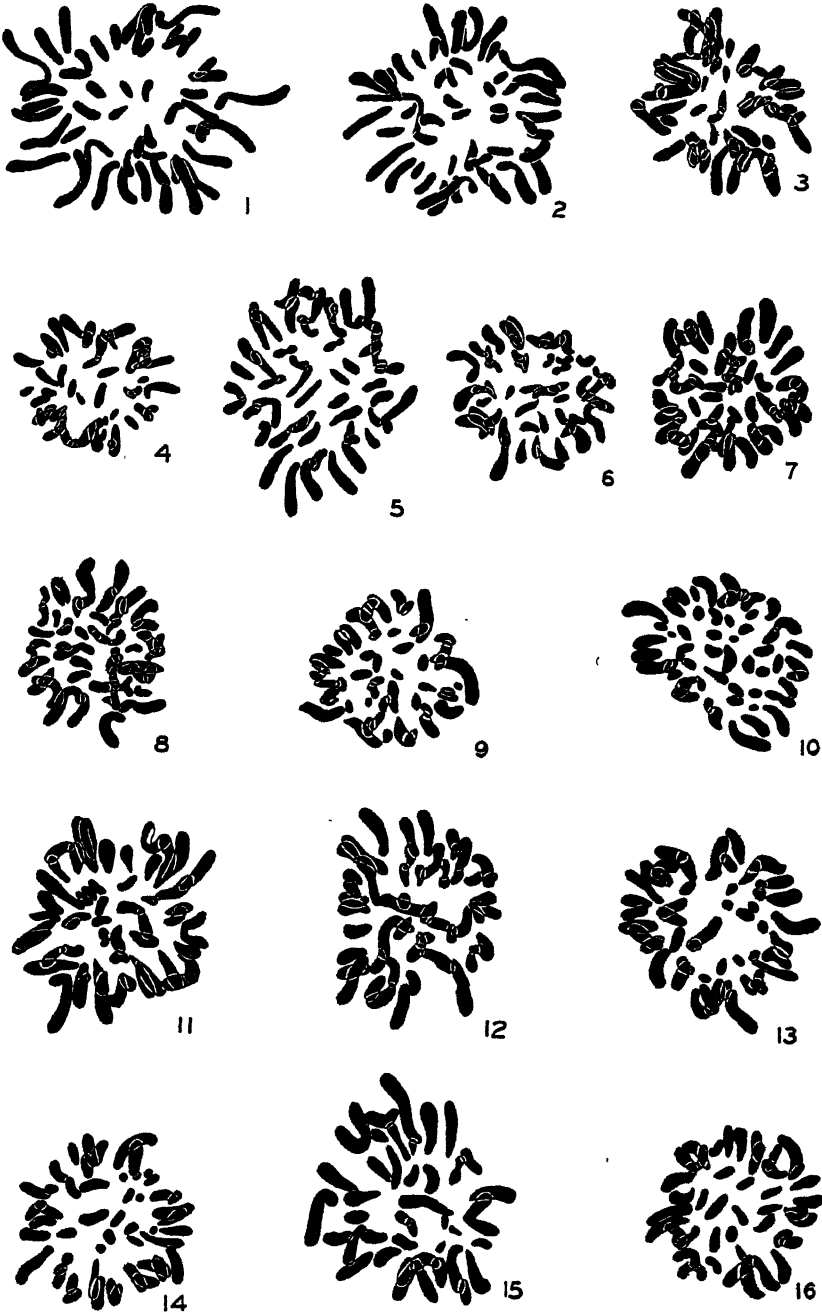
### EXPLANATION OF PLATES

All of the polar views and serial alignments are drawn from metaphase plates of spermatogonia fixed with Allen's modification of Bouin's fluid as described by Painter. The staining was done with Heidenhain's iron-hematoxylin. The magnification of each cell is approximately 4100 diameters.

### PLATE 1

#### EXPLANATION OF FIGURES

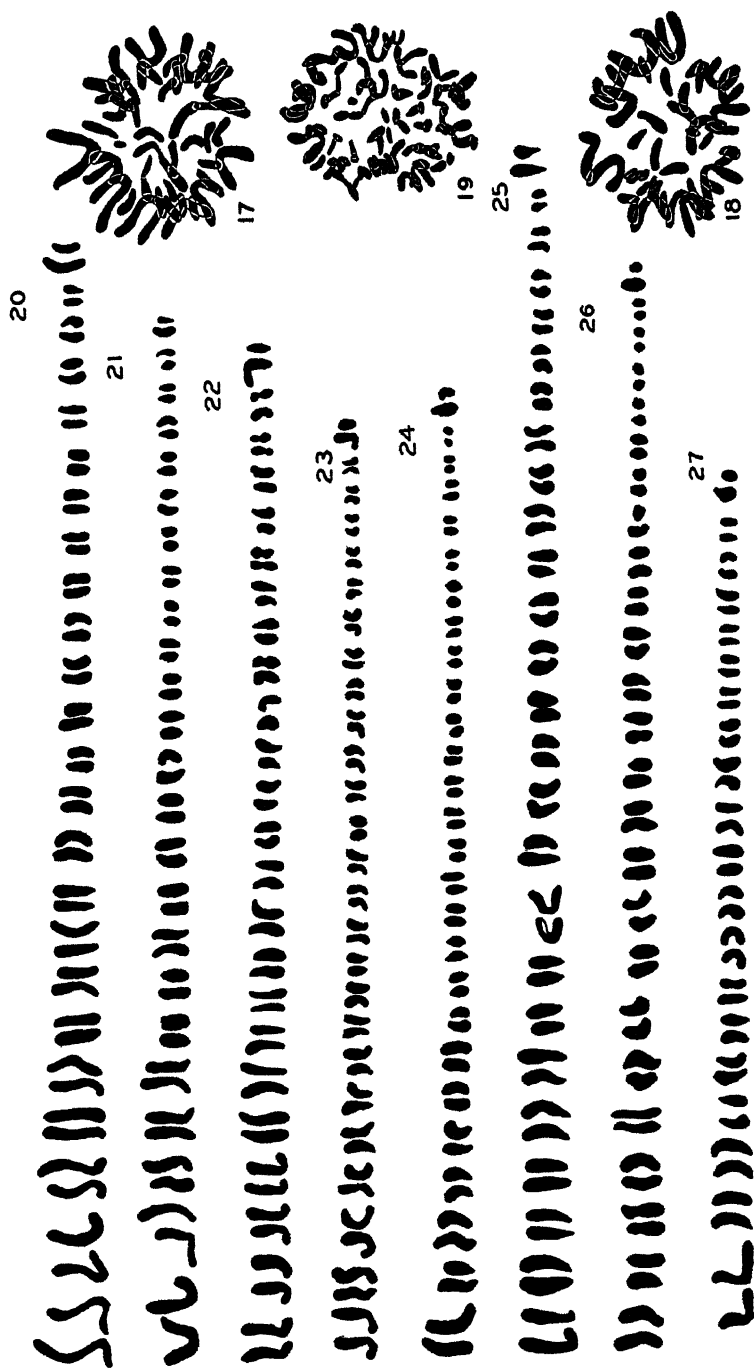
- 1 and 2 *Peromyscus californicus insignis*.
- 3 and 4 *Peromyscus boylei attwateri*.
- 5 and 6 *Peromyscus maniculatus gambeli*.
- 7 and 8 *Peromyscus maniculatus hollesteri*.
- 9 and 10 *Sigmodon hispidus texianus* (cotton rat).
- 11 and 12 *Neotoma floridana attwateri* (wood rat).
- 13 and 14 *Microtus townsendii* (meadow mouse).
- 15 and 16 *Rattus rattus alexandrinus*.



## PLATE 2

### EXPLANATION OF FIGURES

- 17 and 18 *Perognathus fallax fallax* (pocket mouse).  
19 *Dipodomys merriami merriami* (kangaroo rat).  
20 Serial alignment from figure 1.  
21 Serial alignment from figure 3.  
22 Serial alignment from figure 5.  
23 Serial alignment from figure 8.  
24 Serial alignment from figure 9.  
25 Serial alignment from figure 11.  
26 Serial alignment from figure 13.  
27 Serial alignment from figure 16.

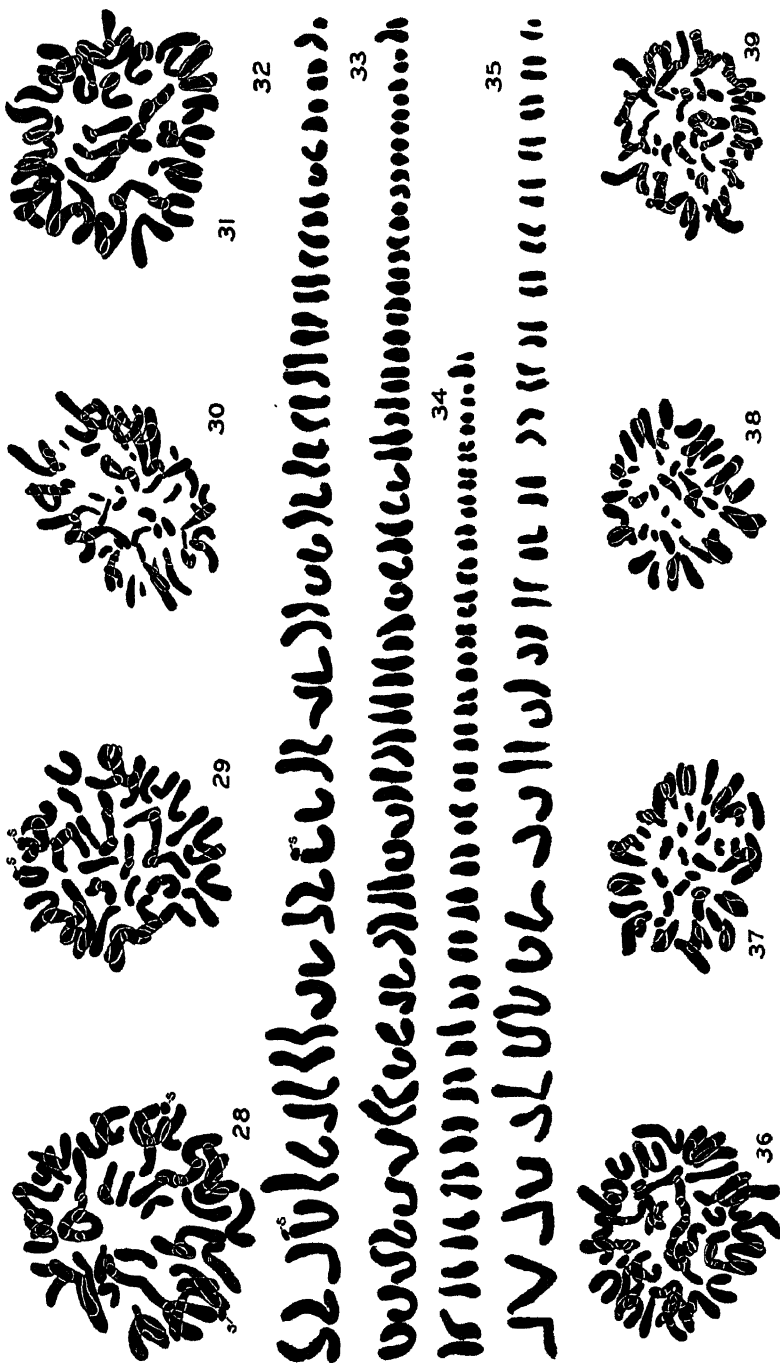


## PLATE 3

### EXPLANATION OF FIGURES

- 28 and 29 *Sciurus carolinensis carolinensis* (gray squirrel).  
30 and 31 *Sciurus niger rufiventer* (western fox squirrel).  
32 Serial alignment of figure 28.  
33 Serial alignment of figure 31.  
34 Serial alignment of figure 37.  
35 Serial alignment of figure 17.  
36 *Sciurus niger limitis* (Texas fox squirrel).  
37 and 38 *Glaucomys volans volans* (flying squirrel).  
39 *Geomys breviceps breviceps* (Louisiana pocket gopher).

CHROMOSOMES OF RODENTS  
JAMES CECIL CROSS





# SPERMATOGENESIS IN STURNUS VULGARIS: REFRACTORY PERIOD AND ACCELERATION IN RELATION TO WAVE LENGTH AND RATE OF INCREASE OF LIGHT RATION<sup>1</sup>

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THREE PLATES (FORTY-EIGHT FIGURES)

## AUTHORS' ABSTRACT

Between December 10th and January 24th, using red, green, and white electric lights at equal intensities of about 1.7 foot-candles, sixty-nine males, with females, were subjected nightly to, a) constant six- and six-and-one-quarter-hour periods of illumination; b) periods increasing gradually from one-quarter to six and one-quarter hours; c) no added light (controls), in a basement room lighted by day from three large windows. Relative heat intensities reaching the birds were: for white, 1; for green, 2.5; for red, 10.

Birds were killed for testis study at 5, 12, 16, 22, and 23 days.

Refractory period was shorter, and modification of spermatogenic activity much greater, under *a* than under *b* types of treatment, as compared with controls. Effects were also more consistent.

Descending order of testis activity induced at all stages was red, white, control, green, under both *a* and *b*.

Effects on testis activity are not proportional to heat intensity of incident light, but depend on wave length, since green effect is not intermediate between those of white and red, as its heat intensity is. Green inhibited germ-cell activity even in birds already at normal midwinter minimal condition.

Constant six-hour red-light treatments, of this intensity, induced first appearance of metamorphosing sperms in twenty-three days in midwinter.

Apparatus and mode of light regulation and measurement are described.

## INTRODUCTION

It has been shown that sexual cycle and spermatogenesis are conditioned by changes in length, intensity, and wave length of daily light ration in the European starling (*Sturnus vulgaris*) and somewhat modified by daily periods of muscular activity. These studies indicated a refractory period for the germinal epithelium and other testis tissues following light changes before these tissues respond to the stimulus occasioned by the light change, and that this refractory period is prolonged by increased daily periods of muscular exercise (Bissonnette, '31 a, b; '32).

<sup>1</sup>Studies on the sexual cycle in birds, no. VII. Aided by a grant from the Committee for Research in Problems of Sex of the National Research Council, 1930-1931.



Rowan ('29, '30, and previous papers), in his studies of this relation of seasonal sexual cycle to light ration, concluded that slow and gradual increases in the daily light period were necessary to give consistent results and that it was the longer period of muscular exercise per day permitted by the longer light periods that really caused the testis changes. In some of Bissonnette's experiments, however, some birds were given sudden increases to maximum light ration without apparent ill effects and with results apparently consistent and in accord with results induced by daily rations which gradually rise to the maximum (Bissonnette, '31 a).

Bissonnette ('32) was led to believe that total white light from incandescent bulbs caused greater acceleration of spermatogenesis in early stages than red light at equal intensity of illumination of 2.6 foot candles, while red was the more effective in later stages and over a longer period. Both red and white proved much more effective in stimulating testis activity than green at all stages at that intensity.

The experiments here described were designed to determine the relative effects upon the length of the refractory period and rate of acceleration of testis activity and the consistency of results with red, green, and white light of, 1) an immediate 6-hour or 6½-hour increase in daily light period, and, 2) a 6½-hour increase reached by small increments of one-quarter to one-half hour per night. These additions of light from incandescent bulbs were added to a normally decreasing day length from December 10th to 21st and to a normally increasing one till January 2nd, in the room previously used (Bissonnette, '30 a, b; '31 a, b; '32).

The refractory period is shorter, acceleration of testis activity greater, and effects more consistent with the first treatment above than with the second. Red is most stimulating, leading to production of sperms in twenty-three days of the constant six-hour dose; white is less stimulating, and green is inhibitory at all stages under both types of dose of light. Stimulating and inhibitory effects of red and green, respectively, on the six-hour dose can be distinguished as

early as five days after the beginning of treatment. These results are not proportional to heat intensity of the incident light, but depend on wave length, the longer red-light waves being stimulating and the shorter green being inhibitory. White is slightly stimulating, though its heat energy is much less than that of the green light used.

#### METHOD

On November 13, 1930, birds were brought into the laboratory and distributed indiscriminately as to sex and age among seven cages, fourteen or more to each cage. They were fed and tended as in previous sets of experiments (Bissonnette, '31 a, b; '32) till December 10th, when experimental work began. Meanwhile necessary measurements of the luminous intensity of the bulbs to be used were made and the distances from the roosts, at which the lights were suspended, determined.

Experience gained in previous experiments led to changes in method of lighting the experimental cages. Interiors of all cages were painted white. Instead of one light and filter in the roof of each cage as used before (Bissonnette, '32), two were used to deliver light directly over the roosts at one-quarter the distance from each end. The mechanical devices for shielding and suspending the light bulbs were the same as used in the previous experiments with filtered colored light, except that the insides of the large galvanized pipes were painted black to prevent reflection of light in them. Filters were also the same red and green ones used before. This double lighting of each cage made the intensity of illumination more uniform within each cage, and lessened the possibility of variations in effect due to lack of uniform intensity. As before, all cages using light treatments had window glass over the light vents, whether they had filters or not. This was necessary with the unfiltered light cages to prevent escape of birds and was used in the other cages for uniformity of conditions, except for the filters. Light reflected from the ceiling was distributed throughout the

room, dimly lighting it. Comparison of inside controls with birds taken from outside in nature showed that it did not compensate for the reduction of daily light in the room as compared with that outside, by day.

One electric time switch was set to give six hours of illumination per night to one red and one green lighted cage throughout the experiment—hereinafter called '6-hour red' and '6-hour green' cages. Another was set each day to give periods, increasing from  $\frac{1}{4}$  hour to  $3\frac{3}{4}$  hours per night, by  $\frac{1}{4}$ -hour increases, by December 24th, and  $\frac{1}{2}$ -hour increases up to  $6\frac{1}{4}$  hours on December 30th, after which it remained constant at that period, to one red, one green, and one white lighted cage, hereinafter called the '15-minute' cages. Controls with no electric light treatments were confined in two cages till January 2nd. After January 2nd and up till January 24th, half of the birds previously kept as controls were transferred to the '15-minute white' cage and run at  $6\frac{1}{4}$  hours' constant light period per night for comparison with the '6-hour reds and greens.' The rest were run as controls.

Samples from controls and from all the 'light' cages were killed for gonad study on December 15th, 22nd, 26th, and January 2nd, at 5, 12, 16, and 23 days. Birds from the '6 $\frac{1}{4}$ -hour white' and the control cage were killed on January 14th and 24th, at 12 and 22 days. These samples usually included juvenile and mature birds of both sexes from each cage. In case all these types were not available, others were taken in their stead. They were all killed in the same way as before, by pressure, and usually died in less than one and one-half minutes without signs of pain other than gaping and convulsive attempts to increase body volume and draw in air.

Gonads were measured, killed, and fixed as in previous studies (Bissonnette, '32) and data on dates of killing, age, gonad dimensions, and microscopic condition, with references to figures in the plates, are included in table 1, for males. Ovaries and oviducts of females were treated in like manner and data recorded for future study. Bodies were stripped of wings, legs, intestines, and feathers, the skin of the neck

TABLE 1  
Data on testes of birds studied

DATE KILLED	TIME AND TYPE OF TREATMENT	AGE OF BIRD	TESTIS DIMENSIONS (RIGHT AND LEFT) IN MILLIMETERS	HISTOLOGICAL DATA. SERIES NO. OF BIRD
	5 days			
Dec. 15	Controls	Y. <sup>1</sup>	R = 2.1 × 1.4 L = 2.6 × 1.1	Figure 1. Midwinter condition, more heavily pigmented than mature bird's testes. B. 13
	Controls	M. <sup>1</sup>	R = 3.0 × 1.9 L = 3.2 × 1.8	Figure 2. Same as above, but with less pigment and few more nuclei in tubules. B. 14
	6-hr. green	M.	R = 3.0 × 1.9 L = 3.8 × 1.5	Figure 3. Tubules smaller than in mature control; nuclei less numerous; vacuoles and necrotic nuclei; regressing. B. 4
	6-hr. green	M.	R = 2.7 × 2.3 L = 3.4 × 1.9	Figure 4. Tubules like controls'; nuclei less numerous; necrotic nuclei indicate regression. B. 5
	15-min. green	Y.	R = 1.7 × 1.4 L = lost or absent	Figure 4. Like controls, but tubules slightly smaller; not as much reduced as '6-hour greens' above. B. 11
	15-min. green	M.	R = 2.6 × 1.8 L = 2.8 × 1.5	Figure 5. Necrotic nuclei indicate regression, but less than in '6-hour greens.' B. 16
	6-hr. red	Y.	R = 2.5 × 1.7 L = 2.6 × 1.7	Figure 6. Slight increase in tubule diameter, and fluid contents and mitoses. Germ-cell nuclei near periphery. B. 3
	6-hr. red	Y.	R = 2.6 × 1.9 L = 3.1 × 1.9	Figure 7. Tubules decidedly larger, with more fluid in them; mitoses not more numerous than above. B. 2
	6-hr. red	M.	R = cut L = 3.5 × 2.1	Figure 8. Tubules smaller than in above young '6-hour reds'; nuclei show increased affinity for stain; more active than controls. B. 1
	15-min. red	Y.	R = 2.3 × 1.4 L = 2.5 × 1.4	Figure 9. Differ from controls only in germ cells' increased affinity for stain; few mitoses; little more active. B. 10
	15-min. red	M.	R = 3.0 × 1.8 L = 3.3 × 1.5	Figure 9. As above '15-minute red.' B. 9
	15-min. white	Y.	R = 2.9 × 1.9 L = 3.0 × 2.1	Figure 10. Few mitoses in tubules; increased affinity for nuclear stain; very slightly accelerated activity. B. 6
	15-min. white	Y.	R = 2.6 × 2.0 L = 3.0 × 1.8	Figure 10. As above. B. 7
	15-min. white	M.	R = 2.5 × 1.8 L = 2.8 × 1.8	Figure 10. As above. B. 8
	12 days			
Dec. 22	Controls	Y.	R = 2.5 × 1.5 L = 2.8 × 1.4	Figure 11. Normal midwinter condition. B. 30
	Controls	M.	R = 3.6 × 2.3 L = 3.8 × 1.9	Figure 12. Tubules not larger than above; so must be longer, since testis is larger. B. 31
	6-hr. green	Y.	R = 2.0 × 1.7 L = 2.4 × 1.4	Figure 13. Tubules smaller than controls'; necrosis not now evident; rare mitoses of germ cells. B. 19
	6-hr. green	M.	R = 3.5 × 2.5 L = 4.4 × 2.1	Figure 14. Tubule diameter slightly larger than controls' or than preceding; germ-cell nuclei more numerous; not inhibited. B. 18
	15-min. green	Y.	R = 1.9 × 1.7 L = 2.0 × 1.5	Figure 15. Fewer nuclei within tubules than in controls; no mitoses; less active than controls, slightly inhibited. B. 28
	15-min. green	Y.	R = 2.6 × 1.8 L = 2.9 × 1.7	Figure 16. Intermediate between the above testis and the one to follow this one. B. 25
	15-min. green	Y.	R = cut L = 3.2 × 1.9	Figure 17. Nuclei more numerous than in control tubules; mitoses present; but not accelerated over controls. B. 29
	15-min. green	M.	R = white <sup>2</sup> L = 3.2 × 2.2 green	Figure 18. Almost not pigmented; tubule smaller than above; necrosis evident. B. 26
				Figure 18. Pigmented normally; otherwise like above. B. 27

<sup>1</sup> Y. = juvenile bird, in first year of life. M. = mature bird, over one year old.<sup>2</sup> Not measured.

TABLE 1—(Continued)

DATE KILLED	TIME AND TYPE OF TREATMENT	AGE OF BIRD	TESTIS DIMENSIONS (RIGHT AND LEFT) IN MILLIMETERS	HISTOLOGICAL DATA. SERIES NO. OF BIRD
	12 days			
Dec. 22	6-hr. red	Y.	R = 4.9 × 3.7 L = 6.0 × 3.3	Figure 20. Tubules much enlarged; more germ-cell nuclei; still only spermatogonia; cytoplasm abundant in tubule. B. 15
	6-hr. red	M.	R = 3.9 × 2.7 L = 5.0 × 2.4	Figure 21. Less changes than the above, but much stimulated so far as cytoplasm of tubules is concerned. B. 16
	6-hr. red	M.	R = 3.4 × 2.8 L = 3.9 × 2.3	Figure 22. Less activated than above, but more than controls. Increased cytoplasm in tubules; secretion (?). B. 17
	15-min. red	Y.	R = 2.4 × 1.9 L = 2.7 × 1.4	Figure 23. In advance of controls; mitoses present, not up to '6-hour reds' above. B. 22
	15-min. red	M.	R = 3.6 × 2.6 L = 4.0 × 2.2	Figure 23. As above. B. 23
	15-min. red	M.	R = 4.0 × 3.4 L = 5.0 × 2.6	Figure 24. Some regression here; necrotic nuclei; possibly roosted in darker part of cage, after being in light. B. 24
Jan. 14	6½-hr. white	Y.	R = 4.4 × 3.3 L = 5.0 × 2.8	Figure 25. Tubules much enlarged; not up to figure 20 of '6-hour red'; syn- zysis present. Germ cells more ad- vanced. One-quarter hour more light per day. B. 32
	6½-hr. white	Y.	R = 2.7 × 1.9 L = cut	Figure 22. Tubules enlarged above con- trols; like least affected '6-hour red' at twelve days. Cytoplasm in tubule much increased; germ cells not much. B. 33
	Controls	Y.	R = 2.8 × 2.0 L = 3.0 × 1.9	Figure 12. Show some advance beyond controls of December 22nd in germ- cell activity, but not in tubule size. Mitoses. B. 35
	Controls	Y.	R = 2.2 × 1.6 L = 2.6 × 1.3	Figure 12. As above. B. 36
	Controls	M.	R = 2.7 × 2.0 L = 3.0 × 1.9	Figure 11. Farther advanced than above young controls; mitoses present; tubules larger; cytoplasm abundant. B. 34
Dec. 22	15-min. white	Y.	R = 2.3 × 1.7 L = 2.5 × 1.4	Figure 26. In advance of controls of this date in germ-cell number and activity; not up to '15-minute reds.' B. 20
	15-min. white	M.	R = 2.9 × 2.0 L = 3.6 × 1.8	Figure 26. As above. B. 21
	16 days			
Dec. 26	Controls	Y.	R = 2.1 × 1.2 L = 2.4 × 1.1	Figure 27. Little if any change from December 22nd; not yet affected by increasing day length. B. 48
	Controls	M.	R = 2.4 × 1.8 L = 3.1 × 1.4	Figure 28. As above. B. 47
	6-hr. green	Y.	R = 1.6 × 1.4 L = 2.0 × 1.4	Figure 27. Testis tubules very slightly smaller than in controls above; germ cells less active; smaller than at twelve days; mitoses rare. B. 40
	6-hr. green	M.	R = 2.7 × 2.5 L = 3.2 × 2.3	Figure 30. Tubules smaller than in above young bird; smaller than at twelve days; mitoses rare. B. 39
	15-min. green	Y.	R = 2.0 × 1.5 L = 2.2 × 1.2	Figure 31. Tubules smaller than controls; but not than '6-hour greens' just above. B. 46
	15-min. green	M.	R = 2.6 × 2.1 L = 3.0 × 1.9	Figure 31. As above. B. 45
	6-hr. red	Y.	R = 4.5 × 3.6 L = cut × 2.7	Figure 32. Tubules much enlarged, 2.6 times control in diameter; numerous syn- zysis stages; spermatocytes. B. 38
	6-hr. red	M.	R = 7.0 × 5.3 L = 8.1 × 4.6	Figure 33. Tubules much enlarged; 4 times control in diameter; at stage of March 20th to April 1st, on normal cycle. B. 37
	15-min. red	Y.	R = 2.4 × 1.9 L = 2.6 × 1.5	Figure 34. Slightly advanced beyond '15- minute reds' of twelve days' treatment; also beyond controls; less than '6-hour reds' of this date. B. 44

TABLE 1—(Continued)

DATE KILLED	TIME AND TYPE OF TREATMENT	AGE OF BIRD	TESTIS DIMENSIONS (RIGHT AND LEFT) IN MILLIMETERS	HISTOLOGICAL DATA. SERIES NO. OF BIRD
16 days				
Dec. 26	15-min. red	M.	R = 2.6 × 1.9 L = 3.4 × 1.7	Figure 34. As above. B. 43
	15-min. white	Y.	R = 1.9 × 1.5 L = 2.4 × 1.4	Figure 35. Slightly in advance of controls, but behind '15-minute reds' above; few mitoses. B. 42
	15-min. white	M.	R = 3.0 × 2.3 L = 4.0 × 2.1	Figure 35. As above. B. 41
23 days				
Jan. 2	Controls	Y.	R = 2.2 × 1.4 L = 2.3 × 1.2	Figure 27. Not changed appreciably since sixteen-day killing. B. 64
	Controls	M.	R = 2.6 × 2.0 L = 3.0 × 1.7	Figure 28. Not changed appreciably since sixteen-day killing. B. 63
	6-hr. green	Y.	R = 1.9 × 1.6 L = 3.0 × 1.3	Figure 28. Slightly smaller tubules than in controls; a few tubules larger, tissues like controls. B. 53
	15-min. green	Y.	R = 2.1 × 1.3 L = cut	Figure 28. As above. B. 59
	15-min. green	M.	R = 2.8 × 1.9 L = 3.2 × 2.0	Figure 28. As above. B. 60
	15-min. green	M.	R = 2.5 × 2.0 L = 2.7 × 1.6	Figure 36. Minimal activity; tubules very small; few nuclei in them; tunica propria thickened. B. 61
	15-min. green	M.	R = 2.7 × 1.5 L = 2.8 × 1.5	Figure 36. As above. B. 62
	6-hr. red	Y.	R = 8.0 × 5.5 L = 9.0 × 5.3	Figure 37. Metamorphosing sperms first appearing; all germ-cell stages present. B. 51
	6-hr. red	Y.	R = 8.0 × 6.7 L = 9.4 × 5.7	Figure 38. Sperm tails just beginning to form; approach conditions of April 1st, on normal cycle; tubules very large. B. 52
	6-hr. red	M.	R = 8.2 × 6.1 L = 10.2 × 5.5	Figure 39. Tubules smaller than above; metamorphosing sperms present. B. 49
	6-hr. red	M.	R = 11.9 × 8.0 L = 12.7 × 7.3	Figure 40. Tubules very large. Young sperms present. B. 50
	15-min. red	Y.	R = 3.3 × 2.2 L = 4.0 × 2.2	Figure 41. Tubules larger than controls; synizesis stages present; mitoses numerous. B. 57
	15-min. red	Y.	R = 3.7 × 2.3 L = 4.0 × 2.1	Figure 42. Like the above, but more germ cells present; same stage otherwise. B. 58
	15-min. red	M.	R = 3.6 × 2.2 L = 4.0 × 1.9	Figure 41. Like B. 57 above. Resemble '6½-hour whites' at twelve days very nearly. B. 56
	15-min. white	Y.	R = 3.1 × 2.0 L = 3.8 × 1.6	Figure 43. In advance of controls, but not up to '15-minute reds'; germ cells active; spermatogonia only. B. 55
	15-min. white	M.	R = 2.7 × 1.7 L = 3.2 × 1.8	Figure 43. As above. B. 54
22 days				
Jan. 24	6½-hr. white	Y.	R = 4.1 × 3.0 L = 5.5 × 2.7	Figure 46. In advance of '15-minute reds' at twenty-three days; synizesis stages numerous; no primary spermatocytes. B. 70
	6½-hr. white	Y.	R = 2.7 × 2.4 L = 3.4 × 2.1	Figure 47. Not quite up to '15-minute reds' at twenty-three days; rare synizesis stages present. B. 71
	Controls	Y.	R = 2.5 × 1.7 L = 2.8 × 1.4	Figure 44. Still at midwinter minimal condition. B. 66
	Controls	Y.	R = 2.9 × 1.9 L = 3.9 × 1.7	Figure 44. As above. B. 67
	Controls	Y.	R = 2.8 × 1.8 L = 2.9 × 1.4	Figure 45. Slightly more advanced than above; more germ cells. B. 68
	Controls	M.	R = cut × 2.0 L = 4.4 × 1.8	Figure 44. Like B. 66 and B. 67 above. B. 69
	Controls	M.	R = cut × 2.0 L = 4.4 × 1.8	Figure 44. Like B. 66 and B. 67 above. B. 69
Jan. 21	Outside control	M.	Not measured	Figure 48. In advance of inside controls, but not up to twenty-three-day '15-minute whites'; germ cells active. B. 65

slit open, and the mouth propped open to admit killing fluid. Each body was then tagged with date, sex, gonad size, time of treatment, and killed and fixed in Bouin's fluid, modified as described before (Bissonnette, '32), for four to five days. They were then washed in alcohol and stored in 80 per cent alcohol for future study of other endocrine glands for possible correlations with the gonads.

Testes were subjected to the usual paraffin technique, cut  $7.5\ \mu$  thick, stained in Heidenhain's iron hematoxylin, without counterstain, and mounted in balsam.

Photomicrographs to the same scale of 335 diameters were made of comparable testis sections showing at least one tubule cut transversely, with its surrounding tunica propria and interstitial tissue. One or both testes of seventy birds were so treated. Prints  $3\frac{1}{4} \times 4\frac{1}{4}$  inches were made from Wratten M panchromatic plates.

In one bird from the '15-minute green' cage, killed at 12 days, one testis (right) was apparently not pigmented, while the other was normally pigmented. Both were sectioned and photomicrographed. In all birds at least half of the left testis was used for sectioning and, when they were very small, both were sectioned.

The intensity of illumination at the roost for each type of light was standardized at 1.7 foot candles by measurement on a 300-cm. optical bench with Lummer Brodhum carriage, and adjustment of distance between light source and roost made accordingly. For white light, a 25-watt bulb at 41 inches; for green, a 100-watt bulb at 36.8 inches, and for red, a 200-watt bulb at 35.6 inches, were used. All lamps were used on the commercial A. C. line in the building and so were subjected to fluctuations of voltage and consequent fluctuations of luminous intensity and of surface illumination.

Tests, with a thermopile in series with a variable-resistance and low-resistance galvanometer, were made of the relative heating effects at the roosts of the different lights as used. Taking the heat effect at the roost from the white light as unity, these heat effects were: white, 1; green, 2.5; red, 10.

It was noticed that the radiation reaching the thermopile in the cages with green filters gradually increased with the time after turning on the light, and persisted for a time after the light was switched off. This indicates that part of the thermal effects were due to reradiation of absorbed radiation. No attempt was made to measure the energy in ergs per unit area per unit time reaching the roost, because the control required for it seemed impracticable with this problem.

## RESULTS<sup>2</sup>

*a.* After five days of treatment (December 10th to 15th), birds subjected to green light (figs. 3, 4, 5) have undergone regression, even from the normal midwinter quiescent condition of germ cells shown by controls (figs. 1, 2). Those subjected to 6-hour constant treatments were more affected than those with '15-minute' gradual increases. The visible signs of this regression consist of vacuoles in the intratubular cytoplasm and necrotic nuclei among germ cells (Bissonnette, '30 b, '31 a). Birds subjected to red light and white light were stimulated to increased testis activity, '6-hour reds' most (figs. 6, 7, 8), '15-minute reds' (fig. 9) and '15-minute whites' (fig. 10), hardly appreciably. This is shown by increase of cytoplasm within the tubules and of nuclear affinity of germ cells for stain. The order of decreasing amount of germ-cell activity is therefore red, white, control, green, with green inhibitory; and the 6-hour constant experimental treatments are more effective than the gradually increasing ones in affecting the testes and overcoming the refractory period.

*b.* At twelve days (December 10th to 22nd, and January 2nd to 14th) the descending order of testis activity on approximately equal time and intensity of experimental illumination, both for the constant 6- and 6½-hour periods and the '15-minute' ones, was 'reds' (figs. 20, 21, 22, 23, 24), 'whites' (figs. 22, 25, 26), controls (figs. 11, 12), 'greens' (figs. 13, 14, 15, 16, 17, 18, 19), except in the case of one '6-hour green' bird (fig. 14), which was similar to most advanced controls.

<sup>2</sup> See table 1 for details.



Again the 6-hour constant experimental illumination periods were more effective than the gradually increasing periods. Green light inhibited testis activity.

c. After sixteen days (December 20th to 26th), as before, 'reds' were most active (figs. 32, 33, 34), 'whites' next (fig. 35), controls still at midwinter normal (figs. 27, 28), and 'greens' inhibited (figs. 27, 30, 31). Effects were still much greater for the 6-hour constant treatments than for the ones gradually increased. 'Six-hour reds' reached the testis condition of March 20th to 31st on the normal seasonal cycle and '6-hour greens' were approaching their minimal condition, below that normal for midwinter.

d. After twenty-two and twenty-three days of treatment (December 10th to January 2nd, and, for 6½-hour whites, from January 2nd to 24th), as before, the descending order of testis activity, as induced by light, is, for red (figs. 37 to 42), for white (figs. 43, 46, 47), for controls (figs. 27, 28, 44, 45), for green (figs. 28, 36), with green light still holding testes at or below the midwinter minimum of activity. At twenty-three days the 6-hour red treatments have induced the appearance of metamorphosing sperms in the testes—a condition reached about April 1st on the normal cycle (Bissonnette and Chapnick, '30). No differences were evident between these 'red light' testes and those reaching the same condition on the normal cycle. Even 6½-hour white treatments are not so effective as 6-hour ones with red, even with increasing daylight periods to help the white. They have induced a condition similar to that of March 15th of the normal cycle.

A bird from outside, killed on January 21st, was beginning to show effects of stimulation by the lengthening days in nature (fig. 48).

The results of 6-hour constant increases in light ration, with all three colors used, were just as consistent as, or rather more so, than those from the gradually increased rations, and far greater with red and white lights. With green light the amount of inhibition was about the same, finally, with both types of light ration increase. Refractory period was shorter with the '6-hour' than with the gradual increases.

The testes of one of the mature '15-minute greens,' killed on December 22nd, at twelve days of treatment, were interesting from the almost total lack of pigment in the right one (fig. 19) and the normal pigmentation of the left (fig. 18). In the apparently white testis, some few and small, widely scattered pigment spots were evident in sections and show in the photomicrograph (fig. 19). Other cases of this asymmetry of testis pigmentation have been recorded and discussed (Bissonnette, '32). No plumage asymmetry of pigmentation was correlated with this testis condition.

This study with more evenly distributed light in the cages shows that even in early stages of light stimulation red light of relatively uniform and long wave length is more stimulating to testis activity than white of equal intensity. Our former conclusion, that white in early stages of treatment was more effective than red, may have resulted from the consideration of birds undergoing greater variations in intensity of illumination, due to the poorer and less uniform light distribution in the cages.

#### DISCUSSION

The experiments just described show that, with the low intensity of 1.7 foot candles, a single large increase of light ration persisted in, representing a larger fraction of the already acting light-ration stimulus, is much more effective (and at least as consistent as, if not more so) than small cumulative increases finally reaching a total ration larger than the single constant ration, in modifying testis condition and sexual cycle. Previous experiments in this laboratory (Bissonnette, '31 a, b; '32) suggested that this might be the case. It is therefore evident that the small gradual increases in daily light ration prevailing in nature are not a necessity for the induction of uniform results in sexual-cycle changes in starlings. Much larger increases are more effective. This is contrary to the conclusions drawn by Rowan ('26) for the junco as the result of his experiments. He believes that effects are inconsistent, with daily increases of fifteen minutes

or more in the length of the light periods, but much more uniform and consistent with increases closely approximating those prevailing in spring in nature.

Our results suggest that it is possible that the Weber-Fechner law, regarding the minimal changes in intensity of stimuli necessary to produce perception of the difference by the organism through its receptors, may be descriptive of this testis reaction to light changes. The changes induced are in some way related to the magnitude of the light-ration change and to its rate of change. The reaction system suggests something of the nature of the 'neuro-humeral' mechanism suggested and described by Parker ('31), in that the light stimulus affects some receptors and stimulates the action of some endocrine gland (hypophysis, thyroid, or adrenal) which may in turn influence the germinal epithelium of the testis toward activity. On the other hand, it may be possible for such rays as can pass the pigmented feathers to act directly upon the germinal epithelium itself, and it may then affect other organs. The increased secretion of fluid within the tubules, noted at twelve days under red-light treatments of six hours' duration (figs. 29, 21, 22), and consistently occurring in early stages of testis stimulation by light, suggests a glandular activity of either Sertoli cells or germ cells, or both, accompanying or preceding germ-cell multiplication.

Ample confirmation has been gained for our previous suggestion that green light, at 2.6 foot candles' intensity, is probably inhibitory in action upon germ-cell activity (Bissonnette, '32). Results just stated show it is inhibitory at 1.7 foot candles, even in birds already at the normal midwinter minimal activity, whether in constant doses of relatively long duration or in gradually increasing doses. Constant larger doses are the more effective. In similar doses red light is stimulating to germ-cell activity. So far as it goes, this is in agreement with the effects of red and green light on the germination of plants (Sheard, Higgins, and Foster, '30). These authors, however, found chlorophyll to develop best under green light, while Sayre ('28) found that, on about

equal energy values, red rays were most effective, green next, and blue least. As to this we cannot say, since our experiments used equal intensity of illumination, and measurements showed that, so far as heat energy is concerned, our light delivered at the roost heat in the proportions: white, 1; green, 2.5, and red, 10. So that red gave four times as much heat energy to the birds as green. However, green is not intermediate in action between red and white. It is inhibitory, while the other two are stimulating to the birds' testes.

The failure of green light of 2.6 foot candles to prevent testis regression on reduced daylight rations, noted by Bissonnette ('32), is shown to be, in part, due to reduced intensity of illumination and also, in part, to inhibitory action of green light itself. Red and white, of the same luminous intensity, were able to prevent or retard regression under like conditions. This also agrees with our results, with those colors, in this study.

Since our last paper went to press we have noted that Parhon, Cahone, and Mârza ('30) have found seasonal variations in the 'hydration' of the testicles in birds as well as in blood and in other organs. This is in accord with the increased fluid contents which we find in the tubules of birds both naturally and experimentally brought to sexual activity and noted above.

Riddle ('31) reports that, if doves or pigeons reach the age of four to five months between February and July, inclusive, sexual maturity is hastened. This is the season when, even in mature birds, the size and activity of the thyroid are decreasing. On the other hand, if they reach this age in autumn, sexual maturity is delayed as much as 36 per cent in pigeons and 52 per cent in doves. He suggests that the anterior hypophysis is also related to this phenomenon. He has also found a seasonal factor in practically all aspects of sex and reproduction studied in doves and pigeons. The period in which sexual maturity is hastened is the one with lengthening days, relatively high intensity of red rays, and

increasing total daily light, while that of delay is the period of regression in starlings' testes, depending on receding light effectiveness. The correlation of the appearance of sexual maturity in doves and pigeons with the demonstrated light stimulation of sex glands in starlings by Bissonnette ('31 a, b; '32) and in juncos and crows by Rowan ('29, '30, and preceding papers) is significant, and suggests light as the conditioning external factor in the seasonal changes in the aspects of sex and reproduction in doves and pigeons as well. Further interest is added by the facts that pigeons breed throughout a relatively long season each year, with repeated climaxes during that period, while doves, starlings, juncos, and crows have relatively short periods of sexual activity per year, with a long period of inactivity. Crows, juncos, and doves migrate in nature, while starlings do not in this country. This has been discussed at greater length elsewhere (Bissonnette, '31 c).

Collier ('31), in his review of the literature on the physiological action of light, shows that the effects of radiation follow the Grotthus-Draper law. Only absorbed radiation has any effect. He finds that the Lambert-Beer law also holds good; the fraction absorbed by a given thickness of protoplasm is independent of the intensity. These hold for the direct effects upon the skin and absorbing surfaces; but it is very likely that more than direct effects are concerned in these testis changes which are in some respects similar to epithelization. The most completely absorbed rays are outside our limits of spectral regions, since the visible rays extend from 7500 to 4000 Å.U., and those most absorbed are between 2000 and 1250 Å.U. However, red visible light has been shown to penetrate several centimeters of tissue, and pigment and circulating blood affect this penetration considerably. Local heating through several centimeters of tissue results, with pigment acting as an absorption screen (Collier, '31). It is here that the heavy green pigment of the intertubular regions of these testes may modify the effects of any rays that pass the pigmented feathers.

Rowan ('26) maintains that it is the longer period of daily muscular exercise that really causes the testis changes in juncos and, by implication, in crows. With this view our findings for the effects of colored lights on testis activity (Bissonnette, '32, and in this study), that red light and white stimulate testis activity, while green of the same luminous intensity inhibits it in starlings, are not in accord. Neither are our results with increased daily exercise periods without extra light or with it. They show, quite conclusively, that, in starlings, added work periods merely prolong the refractory period before light changes can produce their reactions in testes, and do not of themselves cause testis activity. It appears that work cannot of itself be the controlling factor in testis cycles in all birds, even if it may be in some. It appears even less likely to be the factor in migratory birds, for in them the long flight south in autumn would tend to stimulate them to testis activity, leading possibly to a breeding period in their winter quarters. That this does not occur in most migratory birds is a well-known fact. If it were the factor underlying the differences of testis activity of our experimental birds, we should expect to find noticeable differences between the activity of birds in different colored lights. No such differences could be detected, even with careful watching. So we believe increase of exercise periods is not the fundamental cause of increased seasonal sexual activity in these birds, but light of relatively long wave length and increasing intensity and duration daily. The evidence supports the conclusion that, with possible limitations, the greater the proportional initial increase of period or intensity, the greater the response of the sex glands to the change, whether this response be one of accelerated activity or of regression.

That the light is not acting through its heat intensity is shown by the fact that while heat energies for white, green, and red are as 1:2.5:10, respectively, the order of induced activity is red, white, control, green. On the basis of heat energy as the conditioning variable, green should be inter-

mediate between red and white. It is inhibitory, while red and white are activating in action.

#### SUMMARY AND CONCLUSIONS

1. Between December 10th and January 24th, using red, green, and white electric light at about 1.7 foot candles' intensity, sixty-nine males, with females, were subjected to, *a*) constant 6- and 6½-hour nightly periods, *b*) periods of light gradually increasing from ¼ to 6½ hours, *c*) no added light (controls), in a basement room, lighted by day through three large windows. Other conditions were similar for all birds.

2. Relative heat energy reaching the birds from the light sources were: for white, 1; for green, 2.5; for red, 10; as measured by a thermopile.

3. Refractory period was shorter, and modification of germ-cell activity much greater, under *a* than under *b* treatments, as compared with controls *c*, at 5, 12, 16, 22, and 23 days, when gonads were studied.

4. Descending order of stimulation light effects, at all stages, was red, white, control, green, under both types of treatment.

5. The differences in effects produced by red, green, and white lights of the same luminous intensities are not proportional to their heat intensities, which are as 10:2.5:1, respectively, while green light is not only not as stimulating as white for testis activity; it is inhibitory, even for birds already at normal midwinter minimal germ-cell activity. Red and white are stimulating.

6. Constant nightly 6-hour red-light treatments, of about 1.7 foot candles' intensity, induced the first appearance of metamorphosing sperms in twenty-three days in midwinter.

7. Results are more consistent with the 6-hour constant light treatments, with all the colors used, than with treatments gradually increasing to 6½ hours per night. The slow increases of daily light in nature in spring are not necessary for consistent or uniform results, so far as germ-cell activity is concerned, at least with this intensity.

8. A bird with right testis almost devoid of pigment and left normally pigmented is described.

9. Apparatus and methods of light regulation and measurement are described.

10. Probable relation of light cycles to age at which birds first reach sexual maturity is discussed.

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## PLATES

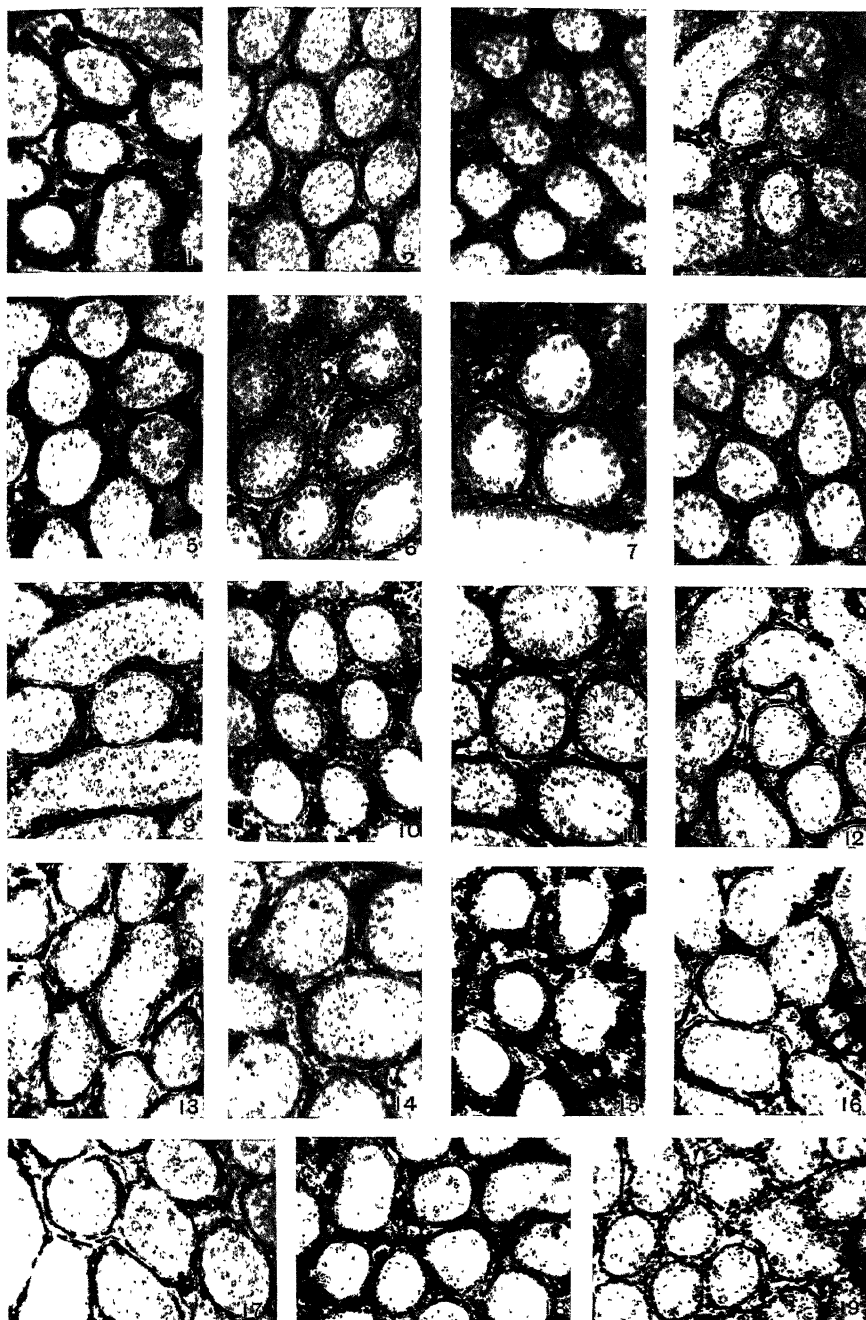
### EXPLANATION OF PLATES

All figures are photomicrographs of comparable testis tubule sections taken in the first instance at 335 diameters and reduced to 223 diameters in reproduction. Typical sections are shown when several testis sections are so much alike as to show no differences appreciable at the reduced magnification. Description of the bird from which the figure is taken is given in each case. Other similar sections coming from birds described in the text are indicated by asterisks (\*).

## PLATE 1

### EXPLANATION OF FIGURES

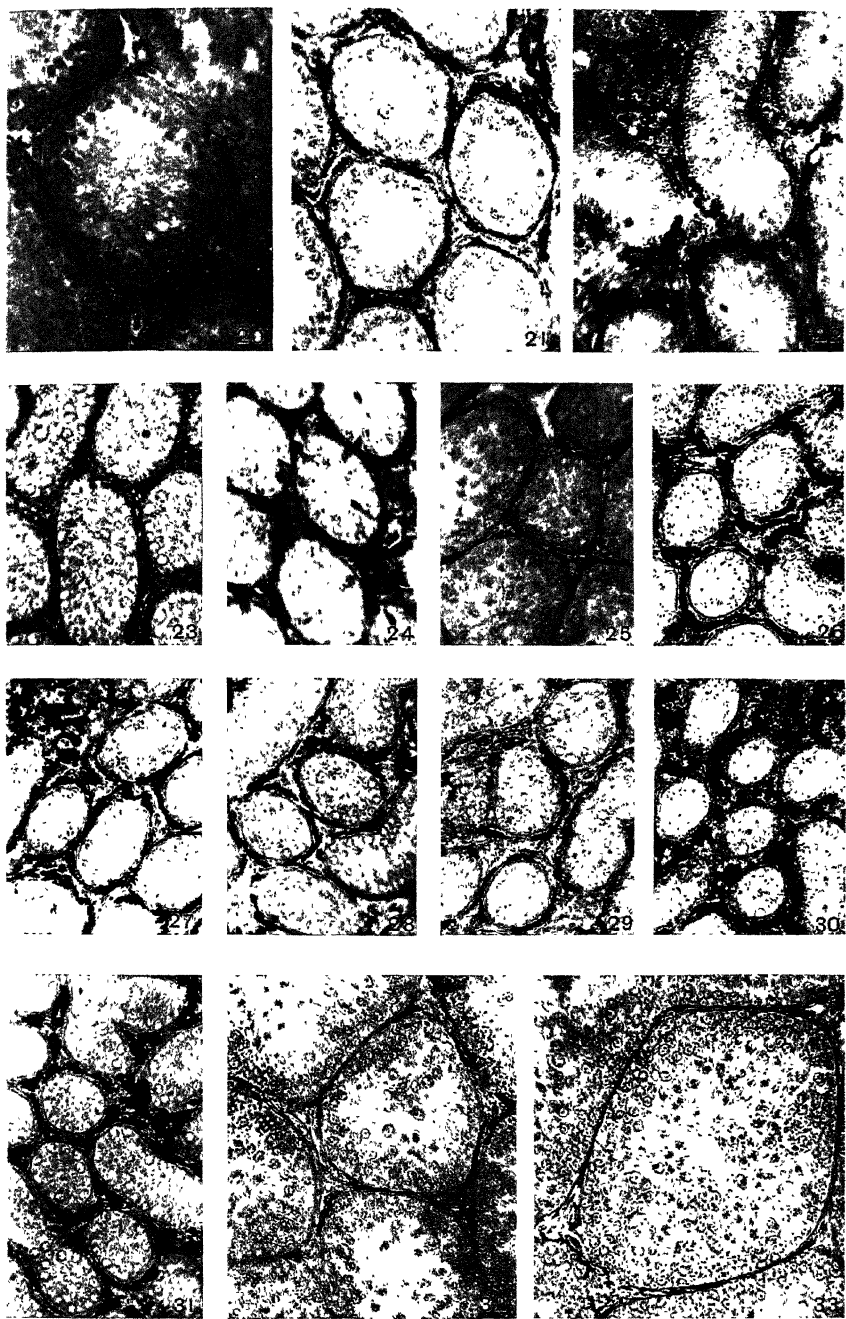
- 1 Young control bird, killed December 15th, at five days. B. 13.
- 2 Mature control bird, killed December 15th, at five days. B. 14.
- 3 Mature '6-hour green' bird, killed December 15th, at five days. B. 4.
- 4 Mature '6-hour green' bird, killed December 15th, at five days. B. 5.
- Young '15-minute green' bird, killed December 15th, at five days.\* B. 11.
- 5 Mature '15-minute green' bird, killed December 15th, at five days. B. 16.
- 6 Young '6-hour red' bird, killed December 15th, at five days. B. 3.
- 7 Young '6-hour red' bird, killed December 15th, at five days. B. 2.
- 8 Mature '6-hour red' bird, killed December 15th, at five days. B. 1.
- 9 Young '15-minute red' bird, killed December 15th, at five days. B. 9.
- Mature '15-minute red' bird, killed December 15th, at five days.\* B. 10.
- 10 Young '15-minute white' bird, killed December 15th, at five days. B. 6.
- Young '15-minute white' bird, killed December 15th, at five days.\* B. 7.
- Mature '15-minute white' bird, killed December 15th, at five days.\* B. 8.
- 11 Young control bird, killed December 22nd, at twelve days.\* B. 30. Mature control bird, killed January 14th, at twelve days. B. 34.
- 12 Mature control bird, killed December 22nd, at twelve days.\* B. 31. Young control bird, killed January 14th, at twelve days.\* B. 35. Young control bird, killed January 14th, at twelve days. B. 36.
- 13 Young '6-hour green' bird, killed December 22nd, at twelve days. B. 19.
- 14 Mature '6-hour green' bird, killed December 22nd, at twelve days. B. 18.
- 15 Young '15-minute green' bird, killed December 22nd, at twelve days. B. 28.
- 16 Young '15-minute green' bird, killed December 22nd, at twelve days. B. 25.
- 17 Young '15-minute green' bird, killed December 22nd, at twelve days. B. 29.
- 18 Mature '15-minute green' bird, killed December 22nd, at twelve days.
- Normally pigmented testis (left). B. 27.
- 19 Right testis of same bird as the above testis (left) came from. This testis is almost without pigment. B. 26.



## PLATE 2

### EXPLANATION OF FIGURES

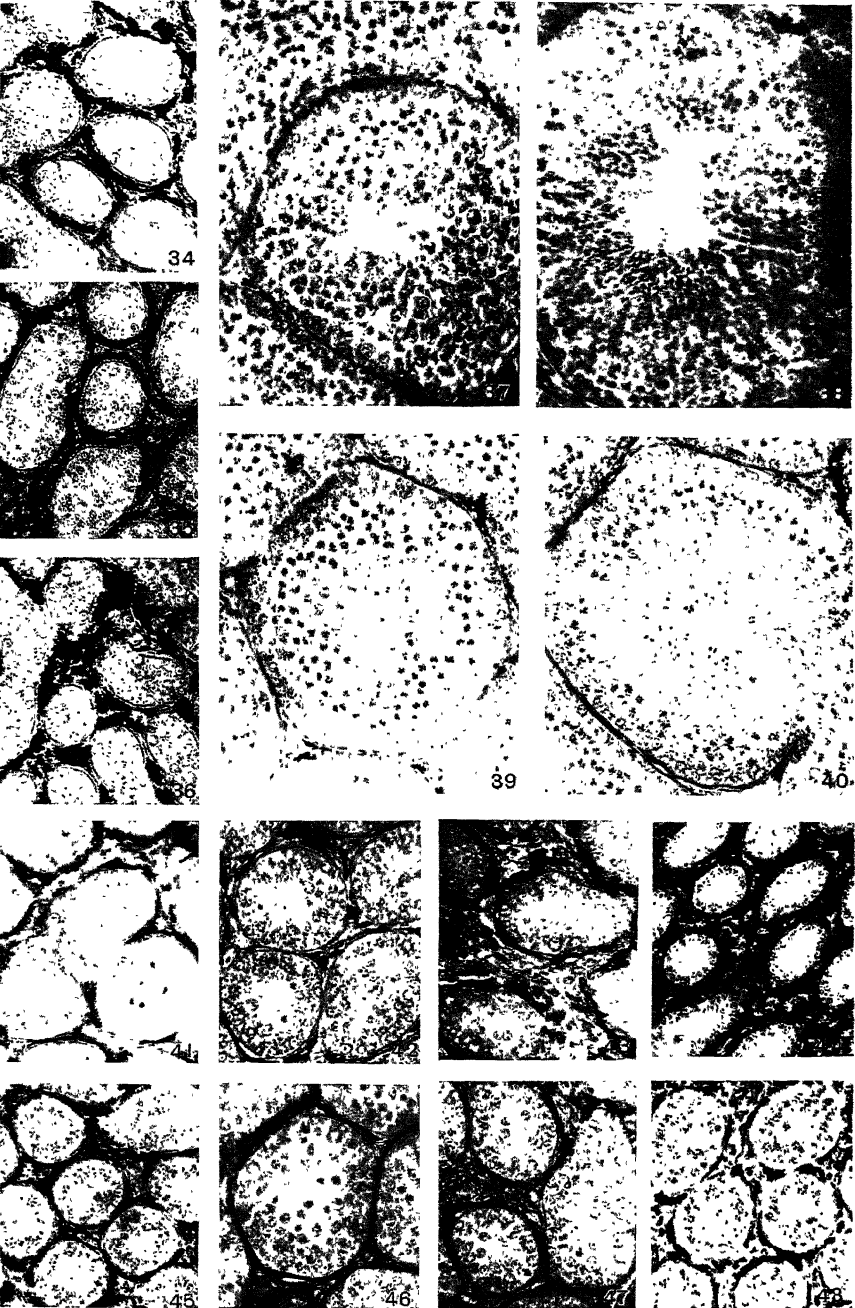
- 20 Young '6-hour red' bird, killed December 22nd, at twelve days. B. 15.
- 21 Mature '6-hour red' bird, killed December 22nd, at twelve days. B. 16.
- 22 Mature '6-hour red' bird, killed December 22nd, at twelve days. B. 17.
- Young '6½-hour white' bird, killed January 14th, at twelve days. B. 33.
- 23 Young '15-minute red' bird, killed December 22nd, at twelve days. B. 22.
- Mature '15-minute red' bird, killed December 22nd, at twelve days. B. 23.
- 24 Mature '15-minute red' bird, killed December 22nd, at twelve days. B. 24.
- 25 Young '6½-hour white' bird, killed January 14th, at twelve days. B. 32.
- 26 Young '15-minute white' bird, killed December 22nd, at twelve days. B. 20.
- Mature '15-minute white' bird, killed December 22nd, at twelve days. B. 21.
- 27 Young control bird, killed December 26th, at sixteen days. B. 48. Young control bird, killed January 2nd, at twenty-three days. B. 64.
- 28 Mature control bird, killed December 26th, at sixteen days. B. 47. Mature control bird, killed January 2nd, at twenty-three days. B. 63. Young '6-hour green' bird, killed January 2nd, at twenty-three days. B. 53. Young '15-minute green' bird, killed January 2nd, at twenty-three days. B. 59. Mature '15-minute green' bird, killed January 2nd, at twenty-three days. B. 60.
- 29 Young '6-hour green' bird, killed December 26th, at sixteen days. B. 40.
- 30 Mature '6-hour green' bird, killed December 26th, at sixteen days. B. 39.
- 31 Young '15-minute green' bird, killed December 26th, at sixteen days. B. 46.
- Mature '15-minute green' bird, killed December 26th, at sixteen days. B. 45.
- 32 Young '6-hour red' bird, killed December 26th, at sixteen days. B. 38.
- 33 Mature '6-hour red' bird, killed December 26th, at sixteen days. B. 37.



## PLATE 3

### EXPLANATION OF FIGURES

- 34 Young '15-minute red' bird, killed December 26th, at sixteen days.\* B. 44.  
Mature '15-minute red' bird, killed December 26th, at sixteen days. B. 43.
- 35 Young '15-minute white' bird, killed December 26th, at sixteen days. B. 42.  
Mature '15-minute white' bird, killed December 26th, at sixteen days.\* B. 41.
- 36 Mature '15-minute green' bird, killed January 2nd, at twenty-three days.\*  
B. 61. Mature '15-minute green' bird, killed January 2nd, at twenty-three days.  
B. 62.
- 37 Young '6-hour red' bird, killed January 2nd, at twenty-three days. B. 51.  
38 Young '6-hour red' bird, killed January 2nd, at twenty-three days. B. 52.  
39 Mature '6-hour red' bird, killed January 2nd, at twenty-three days. B. 49.  
40 Mature '6-hour red' bird, killed January 2nd, at twenty-three days. B. 50.  
41 Young '15-minute red' bird, killed January 2nd, at twenty-three days.\*  
B. 57. Mature '15 minute red' bird, killed January 2nd, at twenty-three days.  
B. 56.
- 42 Young '15-minute red' bird, killed January 2nd, at twenty-three days. B. 58.  
43 Mature '15-minute white' bird, killed January 2nd, at twenty-three days.\*  
B. 54. Young '15-minute white' bird, killed January 2nd, at twenty-three days.  
B. 55.
- 44 Young control bird, killed January 24th, at twenty-two days.\* B. 66.  
Young control bird, killed January 24th, at twenty-two days.\* B. 67. Mature  
control bird, killed January 24th, at twenty-two days. B. 69.
- 45 Young control bird, killed January 24th, at twenty-two days. B. 68.  
46 Young '6½-hour white' bird, killed January 24th, at twenty-two days. B. 70.  
47 Young '6½-hour white' bird, killed January 24th, at twenty-two days. B. 71.  
48 Mature outside control bird, killed January 21st, for comparison with  
inside controls above. B. 65.







# THE DISTRIBUTION OF SPERM-FORMING MATERIALS IN SCORPIONS

EDMUND B. WILSON

SEVEN HELIOTYPE PLATES (EIGHTY FIGURES)

## AUTHOR'S ABSTRACT

There are in scorpions two sharply contrasting types in respect to the mode of distribution of the chondriosomes to the sperm cells. In one of these the chondriosomes, spheroidal in form and nearly definite in number, are sorted out whole without division during the spermatocyte divisions, their number being thus reduced successively to one-half and one-fourth. This type occurs in *Opisthacanthus*, *Hadrurus*, *Vejovis*, *Euscorpius*, and *Palamnaeus*. In the other, as yet known only in *Centrurus*, all the chondriosomes fuse during the spermatocyte growth period to form a single ring-shaped body; and this, during the two ensuing mitoses, is accurately divided into two, four, and eight equal parts, of which each spermatid receives two. In both types alike the chondrioma is thus distributed very nearly equally to the sperm cells, but by widely contrasting processes; and in both types the spermatid chondriosomes are drawn out to form the sheath of the axial filament in the sperm tail. In *Opisthacanthus* there are indications of a definite process of dictyokinesis during the spermatocyte divisions.

These facts are discussed in the light of the general history of the chondriosomes in other animals, with especial reference to more general problems of cell division. The present vagueness and uncertainty of our knowledge of cell division and differentiation are emphasized.

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## INTRODUCTION

Several years ago I briefly reported<sup>1</sup> a new type of chondriokinesis in the sperm-forming divisions of the Arizona scorpion, *Centrurus exilicauda* (Wood), which offers some interesting problems in cell mechanics and, so far as I am aware, is without a parallel in other animals.<sup>2</sup> In the same paper I gave, for the sake of comparison, a short account of the corresponding operations in the California scorpion *Opisthacanthus elatus* (Gervais). A critical comparison of the two cases confronts us with the disconcerting fact that although both the starting point of the process and the final result are essentially the same in the two cases, the intervening stages are of totally different type. I was later able to fortify this result by the study of several other species of scorpions, including *Centrurus henzi* (Banks) from Florida, *C. carolinensis* (Beauv.) from Texas, *Hadrurus hirsutus* (Wood) from Arizona, and *Vejovis boreus* (Wood) from Wyoming.<sup>3</sup> Of these five, the two species of *Centrurus* agree in all essentials with the Arizona form, while *Vejovis* and *Hadrurus* are substantially identical with *Opisthacanthus*. Hereinafter, accordingly, I will designate the two respective cases as the *Centrurus* and the *Opisthacanthus* types. The net result of these studies was to show that in both types the chondriosomes are distributed with almost exact equality, yet in *Opisthacanthus* this result is attained by an apparently random process of sorting out whole chondriosomes, while in *Centrurus* the same result is produced by a fusion of all of

<sup>1</sup>Proc. Nat. Acad. Sci., vol. 2, 1916.

<sup>2</sup>I suspect from Sokoloff's somewhat fragmentary observations on *Buthus* ('13) that a similar condition may be found in that form.

<sup>3</sup>My thanks are due to the well-known authority, Mr. Nathan Banks, of the Museum of Comparative Zoölogy, Cambridge, for identification of the species. I am especially indebted to Prof. D. T. MacDougall and his colleagues at the Desert Laboratory at Tucson for many courtesies extended during my collecting in that region and for sending living material to New York; also to Prof. H. J. Muller for living specimens of *Centrurus* from Texas; to the Southern Biological Supply Company, of New Orleans, for living *Centrurus* from that region, and to the supply department of the Scripps Oceanographic Institute at La Jolla for living specimens of *Opisthacanthus*.

them to produce a regular ring-shaped body which then, like a chromosome tetrad, undergoes two successive equal divisions during the maturation mitoses.

The phenomena in *Centrurus* are of so exceptional a character that I should not have been surprised by a sceptical reception of my earlier brief accounts; in point of fact, only long-continued and repeated study finally removed all doubts concerning the facts. I regret that circumstances have so long delayed an adequate presentation of the evidence. Numerous drawings of both types were long ago prepared, but only a few of them have heretofore been published, and those incidentally in the course of other works.<sup>4</sup> The fuller account here presented is still far from adequate in respect to many interesting questions, but the main facts are perfectly clear; they have been demonstrated repeatedly and in great numbers of cells. I offer here no more than a study of the main facts relating to the chondriosomes, passing over entirely, or with no more than brief mention, many other interesting phenomena that are not seen in my present material clearly enough for satisfactory study. I am convinced that renewed study of the spermatogenesis of both types, with new material and under more favorable conditions, will yield many important further results.

#### METHODS

Owing to the extreme delicacy of the testes, all the methods of fixation tried tend to produce considerable shrinkage, and often overfixation, of the general cytoplasm—in the case of certain stages, indeed, perfect pictures could only rarely be obtained. On the other hand, most of the formed bodies, including the chondriosomes, nuclei, chromosomes, amphiasters, and centrioles, are often well fixed and give very fine pictures. Of many methods tested, the Benda process of fixing and staining (alizarine-crystal violet) proved most successful, giving exceptionally fine pictures of the chondrio-

<sup>4</sup>See "The Cell in Development and Heredity," third edition, 1925-1928, pp. 360, 363, 371. Also, "Protoplasmic Systems and Genetic Continuity," *Am. Naturalist*, vol. 59, pp. 481-496, 1925.

somes and other structures; and this method has been my main reliance for all stages. Less spectacular, but often very clear preparations, showing the cell boundaries and, in later stages, the chondriosomes, are often given by Flemming's fluid followed by iron-haematoxylin, safranin, and other dyes; but after this fixation, both the general cytoplasm and the earlier stages of the chondriosomes are more damaged. Very striking results were obtained by use of Bensley's methyl-green and acid-fuchsin method, the chromosomes being clear green and the chondriosomes bright red. Janus green, employed *intravital*, was also successfully employed. Material fixed especially for the Golgi bodies (methods of Kopsch and of Weigl) gave rather meager results, but were of considerable aid in the study of *Opisthacanthus*. Further studies on the questions here at issue are in progress by Doctor Pollister and myself.

Apart from the overfixation and shrinkage referred to above, the main difficulty has been to determine the serial order of the spermatogonial generations because of the irregular scattering through the testis of cysts of different ages. All the cells in each cyst are always exactly or very nearly in the same condition. Only in case of the spermatocytes, accordingly, could the succession of stages be followed completely.

#### PRELIMINARY OUTLINE

It will be advantageous to introduce the more detailed account by a brief outline of the principal facts. In both types alike the starting point is given by the chondriosomes of the early spermatogenesis, which are small separate bodies, scattered through the cytosomes or, at certain stages, massed toward one pole (pl. 5). In both types the chondriosomes are distributed to the spermatids with almost exact equality, and subsequently, as in so many other animals, they are drawn out around the axial filament to form the sheath of the flagellum (pls. 2 and 4). During the middle stages of spermatogenesis, on the other hand, the mechanism of distribution in the two respective cases offers the following apparent contradiction:

1. In the *Opisthacanthus* type the small chondriosomes of the early stages are in the spermatocytes transformed into much larger spheroidal bodies or chondriospheres (pls. 3, 4) which are of nearly, if not quite, constant number (in *Opisthacanthus*, twenty-four, in agreement with Sokoloff's account of *Euscorpius*) and at no time show the least sign of fusion or division. During each spermatocyte division they retain their identity, seemingly remain quite passive, and are sorted out bodily in such a manner that half of them pass to each pole (pl. 3). The number is thus reduced, first to one-half and then to one-fourth (namely, in *Opisthacanthus* to six and in *Vejovis* to five). The reduced group received by each spermatid now becomes arranged in the form of a complete and symmetrical ring surrounding the axial filament near the base of the nucleus. Thus arises a multiple *nebenkern* closely similar to that found in various other invertebrates (pl. 4 and p. 440). Without fusion of its components this structure now elongates backward around the axial filament to constitute finally the envelope of the sperm tail. The foregoing processes, though somewhat unusual, do not differ widely in principle from those familiar in many other animals, among them the European *Euscorpius carpathicus* as described by Sokoloff ('13), and the East Indian scorpion *Palamnaeus*, of which an account has been given by Gatenby and Bhattacharya ('24) and by Nath ('24).

2. Nothing could in appearance be more widely different from the foregoing process than that offered by the *Centrurus* type (pls. 1 and 2). Here, rather early in the growth period, the small separate chondriosomes of the early spermatocytes progressively fuse until they give rise to a group of much larger, thick, rod-shaped bodies. These become aggregated around the idiozome (pl. 6) and finally fuse to produce a single, ring-shaped body which, like a Saturn's ring, encircles the idiozome lying at the nuclear pole (figs. 64 to 67). This body takes up a position tangentially beside the spindle (pl. 1), is drawn out to an ellipsoidal form, and breaks at each extremity to form two half-rings. In the ensuing division

each of these is divided equally and transversely into two equal rods, two of the products passing into each daughter cell (figs. 8 to 11). Persisting unchanged during the ensuing interkinesis, the two rods in each daughter cell are in turn divided equally and transversely during the second mitosis (pl. 2). The original ring is thus divided into eight equal parts, of which each spermatid receives two. These now come close together in each spermatid to form a bipartite nebenkern which is finally drawn out to form the spiral envelope of the flagellum. The history of the chondriosome ring during the spermatocyte divisions thus bears a certain resemblance to that of a heterotype chromosome ring—a resemblance heightened by the fact that in preparations stained by ordinary nuclear dyes, such as safranin or haematoxylin, the ring stains exactly like the chromosomes and almost as intensely. For such a chromosome ring, in fact, I at first mistook it until its true nature was made evident both by its staining reactions and by its morphological history. Concerning its nature there is not the slightest room for doubt; but the mechanism of its division remains an open question and one of much interest for the more general problems of cell division.

#### THE SPERMATOCYTE DIVISIONS AND SPERM FORMATION

##### *A. Centrurus type*

The history of the chromosomes lies outside the scope of this work, but a few facts regarding them may be mentioned in passing. In *Centrurus exilicauda* the dividing spermatogonia show with perfect clearness twenty-six chromosomes, rounded in form and nearly equal in size (fig. 1). When stained in iron-haematoxylin or safranin, the first spermatocyte metaphases seem at first sight to show fourteen chromosomes (fig. 2), thirteen of which, closely grouped in a flat plate, are slightly elongated, while the remaining one has the form of a large closed ring lying just outside the main group parallel to the spindle. In such preparations all these bodies stain alike, the true nature of the ring being quite

concealed. A totally different appearance is offered after double staining by the Benda method or by Bensley's methyl green-acid fuchsin. In the first case the ring is intensely purple, the chromosomes pale reddish; in the second, the ring is bright red, the chromosomes green. These differences, together with the morphological history, decisively establish the chondriosomal nature of the ring; and still further proof is given by the fact that when treated intravital with dilute Janus green, the ring is stained clear green, while the chromosomes are colorless. The Bensley slides often show in addition to the ring a few smaller separate red bodies, the nature of which has not been determined. In its final position, at metaphase, the ring lies very nearly at the level of the chromosome plate, though with slight deviations (figs. 3, 4). In *C. exilicauda* it is typically circular at this time, usually with quite smooth contours. In *C. henzi* and *carolinensis* the ring is often more widely open and less regular.

1. *The divisions.* The ring maintains its equatorial position without material change until the closing stages of the first division, and even after it has divided into four parts (figs. 4 to 11). There is some variation in the latter process. The first step is a drawing out toward both poles into an ellipsoidal form (figs. 4 to 6)—a process which begins during the metaphase at the time of the general karyokinetic elongation and becomes very marked in the final anaphase (fig. 6). Before the cell constriction has begun, the ring usually breaks transversely at both polar apices to form two curved rods, which soon straighten out and lie parallel to the spindle axis (figs. 7, 8). Finally, as the cell body constricts, the two rods are themselves constricted and transversely divided across their middle points (figs. 9, 10). The ring has thus given rise by transverse division to four very nearly equal parts, two of which pass into each of the resulting second spermatocytes (fig. 11).

In the ensuing interkinesis (fig. 12) the chromosomes remain more or less distinct, rounded in form, and closely aggregated, but only rarely, it would seem, give rise to a vesic-



ular nucleus. The two chondriosomes (two-fourths of the original ring) likewise remain distinct and usually lie near together, though sometimes widely separated. These stages, very numerous in all the preparations, clearly show that at no time do these bodies disappear, fuse, or otherwise lose their identity. In the second mitosis they are again more extended and lie parallel to the axis of the spindle. Most frequently they are rather near together (figs. 14 to 16), but I have seen a considerable number in which they lay nearly or quite on opposite sides of the spindle (fig. 13).

In the anaphases of the second mitosis the rods first shorten somewhat and in some cases show some evidence of incipient division across their middle points (fig. 14). A little later, as the cytosome begins to divide, they become actually constricted (figs. 15, 16) and finally are divided transversely to produce four equal bodies, of which two enter each spermatid, meanwhile shortening to an almost spheroidal form (figs. 17 to 19). The study of numerous preparations has left me uncertain as to whether this process is an autonomous act of division on the part of the chondriosomes, or a passive, mechanical result of the cell constriction, or is due to some other cause. In any case it is a noteworthy fact, confirmed by repeated observation, that before any sign of the cell constriction has appeared, the two rods often show a clearer zone in the equatorial plane and may even show a slight median constriction (fig. 14). As in the case of the first division, therefore, the facts make it doubtful whether the cell constriction is directly responsible for division of the chondriosomes. To this question I will later return (p. 455).

2. *Sperm formation.* Although my material is inadequate for a complete study of the sperm formation, the history of the chondriosomes is shown in spectacular fashion, especially in the Benda slides, where these bodies are at every stage brilliantly stained by the crystal violet. In the young spermatids the two chondriosome bodies, at first nearly spherical, become closely associated and often a little flattened together to form a double body which I will speak of as the

nebenkern (fig. 18). Soon afterward the axial filament is seen extending from the base of the nucleus between the halves of the nebenkern with its outer portion passing freely outside the cytosome (fig. 19). The latter now elongates to a pear shape, with the axial filament making its exit from the pointed narrower end, while the halves of the nebenkern still remain spheroidal and near the nucleus (fig. 20). In the next stage (fig. 21) they are seen as two slightly flattened bands, extending backward on either side of the axial filament. Meanwhile the nucleus has become conical, with the acrosome at its tip.

In succeeding stages, as the cytosome elongates, the two chondriosome bands steadily extend backward and at the same time twist about each other, with the axial filament lying between them (figs. 22, 23). By continuation of this process the sperm finally becomes quite filiform, while the double spiral formed by the chondriosome pair is progressively more closely twisted (figs. 24, 25) in finer and more closely crowded turns until it finally disappears from view, even under the highest powers of the microscope. Thus arises the envelope of the flagellum. In the Benda preparations the successive steps of this process offer a remarkable picture. Even after the turns of the spiral have become so fine as to be indistinguishable, the sperm tails are still intensely violet, in striking contrast to the reddish yellow elongate sperm heads, long after the assembly of the sperms in parallel bundles before their discharge. It seems probable from this that even in the fully ripe sperm the double spiral, though no longer visible, is still present.

### *B. Opisthacanthus type*

As above stated, my principal studies on this type have been made on *Opisthacanthus*, but parallel ones on *Hadrurus* and *Vejovis* gave the same general result, differing only in the fact that in *Vejovis* the spermatocyte number of chondriospheres is about twenty (five to each spermatid) instead of twenty-four. In all three the chromosome number is much

greater than in *Centrurus* and the chromosomes are both more elongate and more varied in size. In *Opisthacanthus* the diploid (spermatogonial) number is about sixty to sixty-two. In both *Hadrurus* and *Vejovis* they are smaller and still more numerous, the haploid number (first spermatocytes) being in both at least fifty and the diploid (uncountable) at least one hundred. Roughly speaking, therefore, the chromosomes of *Opisthacanthus* are about twice, and those of *Hadrurus* and *Vejovis* four or five times, as numerous as those of *Centrurus*.

1. *The divisions.* In the middle growth period the chondriosomes are scattered irregularly through the cytosome in the form of large, spheroidal bodies (figs. 72, 73, 78, 79, etc.), fairly uniform in size, and staining intensely blue-black in iron-haematoxylin, bright purple in crystal violet (Benda method), clear red in acid fuchsin, and clear green in Janus green (intravital). After all these dyes (especially after Benda), they show, more or less clearly, a more chromophilic cortex and a lighter medullary substance—a condition that becomes more pronounced in the later stages (pl. 4) and is especially conspicuous after Flemming-haematoxylin. In the earlier stages it is difficult to count them exactly, but in later stages and in favorable cases it clearly appears that in *Opisthacanthus* their number is close to twenty-four—a result that is fully borne out by their history. In the late prophase, after breakdown of the nuclear membrane (figs. 26, 27), the chromosomes are irregularly massed toward the center of the cell with the chondriospheres lying irregularly around and among them (figs. 26, 27). In the metaphase, which quickly follows (figs. 28, 29), the chromosomes lie in the equatorial plane, extending quite through the substance of the spindle, while the chondriosomes remain as before irregularly scattered through the cytosome outside the spindle.<sup>5</sup>

As the chromosomes draw apart the chondriosomes still remain irregularly scattered around the spindle area, though up to a late stage a few may often be seen in the polar regions

<sup>5</sup> Certain other interesting features of the divisions are reserved to a later page (p. 450).

(figs. 30, 31). During the telophases (fig. 31) it is evident that the chondriospheres are in course of allotment to the daughter cells in nearly equal numbers (i.e., about twelve to each), but this can rarely be determined with exactness, owing to the close crowding of the cells and the delicacy of their membranes at this time. The fact is, however, proved by the final result. During the interkinesis a vesicular nucleus is formed, with the chondriospheres, as before, irregularly scattered about it (fig. 32). At this stage the number 12 can sometimes be accurately counted.

Apart from the smaller number of both chromosomes and chondriosomes, the second division is so nearly a replica of the first as hardly to call for description. In the metaphase (fig. 33) the chondriosomes are, as before, irregularly scattered around the spindle—in the specimen figured already separated into two nearly equal groups by the equatorial plate. The late anaphases and telophases are closely similar, except in size, to those of the first division (figs. 34, 35). In figure 34, which shows all the chondriospheres, it is evident that the latter are about to separate into two groups of six each. The result is clearly seen in the spermatids, in very many of which it is easily to see with diagrammatic clearness that the number of chondriospheres is exactly six, these bodies being at first flattened more or less against the nuclear membrane (fig. 35), but later becoming again spheroidal (pl. 4). Certain variations from this number will be considered a little later.<sup>6</sup>

<sup>6</sup> Some of the best Benda slides offer a magnificent demonstration of the polar granules in the chromosomes, situated exactly at the point of attachment (always terminal) to the spindle. When differentiated to precisely the right point, the chromosomes appear pale reddish or yellowish red, the polar granules and chondriospheres deep violet, and the general cytoplasm pale (fig. 29, which is typical of a large number studied, except that for the sake of clearness only a few of the chromosomes are shown). It is clearly seen, further, that from each polar granule a conspicuous 'spindle fiber' extends out to the centriole at the astral center, the appearance being precisely that of two widely separated centrioles connected by a spindle fiber. This condition is seen in both divisions and in hundreds of cells, but only a few of my slides show it with irreproachable clearness. Whether the polar granules have anything to do with the 'trabants' or 'satellites' of plant chromosomes remains to be seen.

2. *Sperm formation.* The foregoing account is based on the study of a large number of preparations, for the most part prepared by the Benda process of fixing and staining (for which this material is exceptionally favorable); but excellent pictures are also often given by the Flemming-Heidenhain method. So far as the chondriosomes are concerned, the same may be said of the following account of the sperm formation.

While the spermatid is still spheroidal the axial filament grows forth from a blepharoplast-like body at the base of the nucleus (fig. 36)<sup>7</sup> while the acrosome is seen in process of formation at the anterior pole. As in *Centrurus*, the cell now becomes pear-shaped, with the axial filament issuing from its narrow posterior end (figs. 37 to 39). It is now easy to see that in a high percentage of cases the spermatid contains six chondriospheres (not all shown in the earlier figures). These are at first irregularly grouped. A little later the nucleus becomes barrel-shaped and later nearly cylindrical (figs. 38 to 40), the acrosome is enlarged and more conspicuous, while the chondriosomes group themselves with schematic regularity in a ring-shaped group surrounding transversely the axial filament at the base of the nucleus (figs. 39 to 41). At this stage, as earlier indicated, the chondriosome ring resembles closely the 'Nebenkernorgan' of Retzius, which persists permanently as the middle piece in the mature sperms of certain invertebrates, including Coelenterates (*Tealia*), Nemertines (*Carinella*), Annelida (*Glycera*), mollusks (*Chiton*, *Mytilus*), and others, and seems to appear in the sperm formation of certain teleosts.

At this stage the spermatids, after successful staining by the Benda method, offer extremely fine pictures, the nucleus being cinnamon red, the chondriosome ring bright purple, and the general cytoplasm pale. The later stages may thus

<sup>7</sup>In later stages a similar body is seen as a disc-like structure lying between the nebenkern and the nucleus and usually staining deeply in iron-haematoxylin (figs. 40 to 45). This body apparently corresponds to Bowen's 'pseudoblepharoplast' and perhaps to Gatenby's 'post-nuclear body.' Possibly it may be derived from the material of the 'polar caps' (Golgi material ?) shown in figures 28, 30, 32, 35.

easily be followed, all regions of the sperm steadily elongating, both nucleus and flagellum becoming cylindrical and finally filiform (figs. 42 to 47). As the action proceeds the chondriosome ring is drawn out bodily to form a sheath which at first encircles the axial filament just behind the nucleus (figs. 38 to 43), but later extends throughout the whole length of the flagellum as far as the end piece. In the earlier stages of this process, at least, there is not the slightest sign of a twisting comparable to that seen in *Centrurus*. The chondriosomes are simply drawn out, first into a spindle shape (figs. 45 and 46) and finally into long filaments lying side by side in close association but up to a fairly late stage not undergoing fusion, their separate identity being revealed by the pale medullary substance, which does not participate in the elongation, but persists for a considerable time near the middle of the flagellum (figs. 46, 47). I have never seen the least indication of a fragmentation of the chondriospheres, such as is indicated by Sokoloff's work ('13) on *Euscorpius*, and by that of Nath ('24) on a species of *Palamnaeus*. The latter observer suggests, probably correctly, that such a process may be an artificial product of fixation.

When viewed axially, the young spermatids, as shown in figure 41, a to c, offer schematically clear pictures of the chondriosome ring. Such views, of which I have examined many hundreds, prove conclusively that the typical number of chondriospheres received by the sperms is nearly constant, namely, in *Opisthacanthus* and *Hadrurus*, six, and in *Vejovis*, five. A slight variation does exist in each species, but in my experience never beyond one more or one fewer than the type. I have studied this most carefully in *Opisthacanthus*, where a count of 500 cases gave (to the nearest whole number) 76 per cent with six chondriospheres, 17 per cent with five, and 7 per cent with seven. In *Hadrurus*, out of fifty-four cases, 64 per cent were found with six chondriospheres and 32 per cent with five, and two or three cases with only one or two, the latter due, no doubt, to accidents of the sectioning or to pathological conditions. In *Vejovis* (of which only a

few were examined) only five chondriosomes were commonly found, rarely four.

#### THE SPERMATOGONIA

In view of the contrast between the *Centrurus* and the *Opisthacanthus* types during maturation, we turn with curiosity to the chondriosomes of the spermatogonia. Owing to the irregular scattering of cysts of different ages in the testis, and for other reasons, I have not been able to trace very fully the succession of spermatogonial stages; but it is perfectly clear that in respect to the chondriosomes there is nothing in these cells to foreshadow the astonishing contrast displayed in those of the spermatocytes. Both types take their point of departure from conditions of essentially the same type, as will at once be clear upon comparison of figures 49 (*Vejovis*), 50 (*Opisthacanthus*), and 51 (*Centrurus*).

In what I take to be the earliest stages (which always lie at or near the periphery of the testis), the cells are roughly pyramidal, with large nuclei, scanty cytoplasm, and are arranged rosette fashion in the cyst, with their apices inward (fig. 47). Toward the center of the cyst the cytosome is nearly filled with small closely crowded chondriosomes, which have the form of delicate filaments, rods, and minute granules; and a few such bodies may often be seen also in the more peripheral regions. These stages have been examined closely only in *Opisthacanthus*, but I believe occur also in the others. The later stages present several well-marked types which, for the reasons above stated, I am not yet able to place in their proper order of succession. All the cells of each cyst are always very nearly at the same stage.

Judging by the chondriosomes, figure 48 may be a dividing cell of the same type as figure 47, but its greater size and somewhat coarser chondriosomes make this somewhat doubtful. Figures 49 to 51 show a type having rather small chondriosomes, short rod-shaped or spheroidal, some of which may be in process of fragmentation.<sup>8</sup> As will be seen, these are

<sup>8</sup> Wilson, *Am. Naturalist*, vol. 59, 1925.

all of quite similar type, though figure 49 is from Vejovis, 50 from *Opisthacanthus*, and 51 from *Centrurus* (all to the same scale). Figure 52 shows a type rather common in *Opisthacanthus*, characterized by very large cells, with enormous nuclei and chondriosomes, and often showing no perceptible membrane. Figure 53 shows an anaphase of what is probably the same type, characterized by very large chondriosomes of various forms and size, but predominantly appearing as rather long coarse threads. I have been unable to connect these forms with the smaller ones that precede them.

Interesting questions concerning the possible genetic continuity of the chondrioma are raised by their behavior in the spermatogonia. This subject has been discussed by many writers since the early work of Altmann, Benda, and Meves,<sup>9</sup> and I can here add little to it. It is obvious that in the testis as a whole the total number of chondriosomes steadily increases as growth proceeds; that at certain stages they increase in number by fragmentation, both in the large and the small spermatogonia; that at every mitosis chondriosomes pass on from mother cell to daughter cells. It should, however, be emphasized that the process of fragmentation is not in general synchronized with the operations of mitosis—it is, indeed, seen at least as often during the interkinesis (fig. 52). as during mitosis. All the evidence goes to show that during the spermatogonial mitoses, alike in *Opisthacanthus* and *Centrurus*, the chondriosomes are simply sorted out bodily, and at random, quite as is the case in the spermatocyte divisions in the *Opisthacanthus* type.

The uncertain point is whether the chondriosomes may also arise *de novo*, as a considerable number of observers have maintained. It must be said that little or no evidence in favor of such a process has been seen in the spermatogonial cells. In the (supposedly) earlier cells (fig. 47) the chondriosomes are too small and crowded for accurate study. In the later ones, whether small or large (figs. 49 to 53) one does not see a series of granules graduating down to the limit of vision,

<sup>9</sup> See, for instance, Duesberg ('10), Bowen ('20), Wilson ('25).



but a fairly definite minimal size below which only an occasional smaller granule can here and there be seen. Whatever the outcome of the issue here raised, it seems probable that some, at least, of the chondriosome material of the spermatocytes and sperms is derived by direct transmission from the earlier generations of spermatogonia, perhaps from still earlier cell generations. I think therefore that, in these cells at least, we must reckon with the possibility, indicated in my earlier paper ('25), that the chondriosome material may conform to the principle of genetic continuity from cell to cell, even though the chondriosomes be not definitely individualized bodies.

#### THE SPERMATOCYTES. GROWTH PERIOD

##### *1. The early spermatocytes*

For the reasons already stated I have not found it possible to follow the final generation of spermatogonia into the youngest spermatocytes with entire certainty. It is clear, however, that the latter, both in *Centrurus* and *Opisthacanthus*, are small cells (in *Centrurus* very small), always found in cysts at or near the periphery of the testis, and in slightly older cysts, graduating by intermediate conditions into indubitable spermatocytes. The cells in question are characterized by very large nuclei, surrounded by a very small amount of cytoplasm, and with the chondriosomes closely massed at one pole (figs. 54, 55). In the latter respect these cells resemble the early spermatogonia as above described (fig. 47); but otherwise are very different. They are smaller, never show the rosette-like grouping, and are conspicuously different in respect to the chondriosomes, which are larger, less numerous, and less elongate. This is most striking in *Centrurus* (figs. 54 to 59), less so in *Opisthacanthus* (fig. 60); but in both cases they differ conspicuously from those of the early spermatogonia shown in figure 47.

In polar views of these cells in *Centrurus* at this stage, or slightly later, the chondriosomes can sometimes clearly be seen to vary in shape from spheroids to rather short rods

and also to vary conspicuously in size (fig. 58, a, b). In the two cases figured their number is thirteen and fifteen, respectively; but they are often too crowded to be counted. I have been unable to identify stages as early as this in *Opisthacanthus*; but a little later (fig. 60) the chondriosomes are longer and more slender and so closely massed that their number cannot be counted. In neither case could an idiozome be distinguished at this time; but such a structure is soon visible in *Opisthacanthus* (figs. 70, 71, etc.) and at a later period in *Centrurus* (figs. 61, 62).

## *2. Chondriosomes during the middle and late growth period*

*A. Centrurus type.* From the early spermatocytes of *Centrurus*, as just described, it is easy to trace a continuous series up to the middle and late growth period. Immediately after the stages just described the two types diverge from each other in a very conspicuous manner as follows. In *Opisthacanthus* the polar massing of the chondriosomes is quickly lost by a scattering of these bodies through the cytosome (figs. 68 to 70); and in this condition they remain, as has earlier been described, until both spermatocyte divisions have been completed. In *Centrurus*, on the other hand, such a process never takes place, the polar aggregation persisting in its original position and finally fusing to form the single chondriosome ring that undergoes division at the time of meiosis. In this respect *Centrurus* differs, so far as I am aware, from all other animals. The remarkable difference between the two types is thus inaugurated almost at the beginning of the growth period.

In the stages which follow in *Centrurus* the cells enlarge, the amount of cytoplasm rapidly increases, while the chondriosomes become larger, elongate, and begin to fuse together, always retaining their position near the nuclear pole. This latter conclusion is placed beyond doubt by the fact that in the polarized bouquet stage of the synaptic period the enlarged chondriosome group always is found at the point toward which the amphitene or pachytene threads converge

(figs. 61, 62) and at its center lies the idiozome. At this time the number of chondriosomes has begun to diminish by fusion, though the actual formation of the ring is not yet fairly under way. Very striking pictures of these stages are given by double staining. With Bensley's methyl-green acid-fuchsin method the nuclear threads are clear, delicate green, the chondriosomes bright red (figs. 61, 62).<sup>10</sup> After Benda's alizarin-crystal violet the nucleus is pale reddish, the chondriosomes deep purple, while the idiozome, now very clearly shown, is light purplish with a darker peripheral layer. After iron haematoxylin the chondriosomes are blue-black, the nucleus and idiozome paler; and in these slides the idiozome often shows in its interior a pair of minute granules that are probably the centrioles (compare figs. 64 to 67, 72, 73).

Now follows a rapid and remarkable process of fusion and remodeling by which the chondriosome group is transformed into a single ring which, when fully formed, gives little or no indication of its composite origin. Figures 63, a to k, show some of the many forms observed during this process in *Centrurus henzi* and figures 64 to 67, the completed ring in *C. exilicauda*. The ring formation is essentially the same in both species except (as above noted) that in the last-named species it is commonly a little smaller, with smoother contours, more regular in shape, and more homogeneous in texture; but I am not wholly certain as to how far these differences may be due to variations in the technique. In both cases the ring shows the same staining reactions as the chondriosomes from which it arises, and stains clear green in Janus green B used intravital. These reactions, together with the morphological history of the ring, leave no possible doubt concerning its chondriosomal nature.

From the conditions shown in figures 63, a to k (which are selected from a much larger number of sketches), it would seem that as the original rod-like chondriosomes diminish in

<sup>10</sup> From a preparation stained by the Bensley method. These figures were drawn in colors with great care by Miss Mabel Hedge, an accurate and experienced observer, and redrawn by the writer in black and white for this work.

number they become associated more or less side by side, flexed more or less into a U shape, and later close to form a complete ring. In the later stages of this process, the incipient rings show a great variety of shapes, including twisted, double, and even interlocking forms (fig. 63, g); but in the end all seem to give the same result.

*B. Opisthacanthus type.* I have not been able to identify the youngest spermatocytes of *Opisthacanthus* with certainty, the earliest distinguishable stage being shown in figure 60. At this time the cells are still very small and the nuclei so closely crowded that the cell boundaries cannot be clearly distinguished. The chondriosomes, rather closely massed toward one pole, are for the most part rod-shaped (some perhaps spheroidal), but are more slender than in *Centrurus*. In the stages that immediately follow the chondriosomes begin to scatter through the cell, at the same time becoming shorter and thicker, the cytoplasm increases in amount, and the cell boundaries become clearly visible (figs. 68, 69). The polar massing of the chondriosomes is thus broken up; and now, for the first time, may be seen (in Benda-haematoxylin material) a body closely applied to the nucleus which I believe to be the idiozome (*i*, in figure 70) and in earlier stages is no doubt concealed by the massing of the chondriosomes. Even at this stage a few chondriosomes may already have become spheroidal (fig. 69). Later all stages may be seen in the transformation of the original rods into small chondriospheres (figs. 70, 71), the latter agreeing closely with the figures of Gatenby and Bhattacharya ('24) and those of Nath ('25) of the Indian scorpion *Palamnaeus*. These are finally converted by simple growth into large chondriospheres, scattered at random through the cell, which persist in this form until their final delivery to the spermatids. I have been unable to find any clear evidence of division, fusion, or new formation on their part; and I believe that their number must be fixed from an early period. That number, as early stated, is approximately (and, I incline to think, exactly) twenty-four; but it is difficult to determine this with certainty.

Figure 72 shows twenty-three; figure 78, twenty; but it is probable that in each case the seeming lack is due to the difficulty of obtaining a clear view of all the chondriosomes in a single section. It is probable that in all cases these bodies consist of a lighter medullary ('chromophobic') and a denser cortical ('chromophilic') substance, as seen more clearly in the later stages; but in strongly stained material this is often scarcely visible.

In *Palamnaeus bengalensis* Gatenby and Bhattacharya figure supposed division stages of the chondriosomes and also a stage in which the chondriospheres of the spermatocytes are loosely massed at one side of the nucleus (their fig. 4) presumably around an idiozome, though such a body is only shown at a later stage when the chondriospheres are again scattered. In *Opisthacanthus* a similar stage is seen, though rarely (figs. 72, 78); but a close massing of the chondriospheres, such as occurs in the early spermatocytes, has never come under my observation in the later stages. To this, as I shall later try to show, is perhaps traceable the astonishing general contrast between the *Centrurus* and *Opisthacanthus* types.

3. *The idiozome. Golgi bodies.* Under this heading I will describe the structure often called the 'idiozome,' perhaps more appropriately the 'idiozome complex,' since it includes what may be the Golgi apparatus and is sometimes spoken of simply as such (e.g., by Gatenby and Bhattacharya). My material is inadequate for a complete account of this structure; but certain observations will be reported here and in the following section that point to some interesting conditions, both in the growth period and during the divisions. In both *Centrurus* and *Opisthacanthus* an undoubted idiozome (or idiozome complex) is present in the spermatocytes from an early period, lying close to the nucleus and exactly at its pole, as is clearly seen during the polarized 'bouquet' or amphitene stage (figs. 61, 62, 73 to 75). In both cases, after suitable treatment, the idiozome sometimes contains a pair of minute granules which are probably centrioles, though I am not wholly sure of this interpretation.

In *Opisthacanthus*, where the facts are clearest, the aspect of the idiozome (or idiozome complex) differs remarkably with the technique. After Benda fixation and staining, it appears as a pale, rounded body, lying at the nuclear pole, with smooth contours, and showing a very delicate, more deeply staining cortical layer (figs. 72 and 73). When stained with iron-haematoxylin, on the other hand, after either Benda or Flemming fixation, the idiozome offers a widely different aspect, being deep black, rough in contour, almost always flattened against the nuclear membrane, and having the appearance of a mass of deeply staining small rods or short threads. This is fairly well seen in the young spermatocytes shortly after the scattering of the chondriosome (fig. 70, Benda-haematoxylin), but is much more conspicuous in later stages (figs. 71, 74 to 77, Flemming-haematoxylin). Some of these clearly show the bodies in question as having an elongate form (fig. 76).

These facts probably mean, I think, that the bodies in question in *Opisthacanthus* form the envelope of a rounded idiozome which is thus concealed from view; that after the Benda method these bodies are unstained—often perhaps destroyed by the fixation—so that the idiozome becomes visible. These facts point to the probability that the bodies in question are nothing other than Golgi bodies, probably somewhat damaged by the fixation, but giving a picture similar to the Golgi bodies of the European scorpion *Palamnaeus* as figured by Gatenby or Bhattacharya ('24); and this is borne out by the fact that in material treated by the Weigl method the idiozome appears deep black. The interest of this conclusion will more clearly appear after consideration of certain phenomena in the ensuing divisions.

In *Centrurus* the case is not so strong, though some interesting suggestions are offered. Here the idiozome complex always lies near the nuclear pole just below the center of the chondriosome ring (figs. 64 to 67). Its appearance varies considerably, depending, I believe, on the technique. In some cases it has the form of a rather vague, pale, rounded body,

with fairly even contour (figs. 64, 65). In other cases—better fixed, I believe—it appears as an irregularly rounded group of separate bodies, which have the form of rather coarse, curved, or contorted and lightly staining threads (possibly plate-like), among which may occasionally be seen a pair of centriole-like bodies (figs. 66 and 67). The thread-like bodies recall the ‘pseudochromosomes’ described by Heidenhain ('00) in the spermatocytes of *Proteus*, the ‘dictyosomes’ of Perroncito ('09), the ‘peri-idiozomic formations’ of Terni ('04), and the like, now generally regarded as Golgi bodies and sometimes as modified chondriosomes (lepidosomes of Parat). In the case of *Centrurus* both the staining reactions and the morphological relations prove that the bodies in question do not belong to the chondrioma (which is represented by the chondriosome ring). I therefore accept the probability that these problematical structures are Golgi bodies, though a few attempts to test this by use of the Kopsch and Weigl osmic methods gave no definite result. It is hoped to reach a more decisive conclusion when new material can be obtained.

4. *The prophases.* In *Opisthacanthus* material prepared by the Flemming-haematoxylin method the prophases and later stages show certain interesting appearances which I suspect may indicate a process of dictyokinesis in some respects more definite than any thus far observed. Two early prophases are shown in figures 79 and 80, in the first of which the nucleus is in a late pachytene, while in the second the breakdown of the nuclear membrane is approaching (the clear zone surrounding the nuclei is probably due to shrinkage). In neither case can the idiozome complex be certainly distinguished, but in its place appear certain masses of a deeply staining material which I suspect to be Golgi material, probably damaged by the fixation. These masses offer an appearance of fragmentation, but I have not yet been able satisfactorily to close the gap between these stages and the interesting ones shown in figures 26 and 27 (compare p. 438). In the latter the nuclear membrane has disappeared, the chromo-

somes lie free in the cytoplasm, and the amphiaster is in course of formation. Two conspicuous centrioles are present, surrounded by rays only slightly developed peripherally but much stronger centrally, to form the apices of the developing spindle. These characters have been seen in many cells, after both the Benda and the Flemming fixation followed by iron haematoxylin. I would especially emphasize the clearness with which the centrioles are seen at this time, for in the Benda slides this appearance is very soon to be changed.

In slides prepared by the Benda method of fixation and staining, as was the case in earlier stages, no trace of Golgi bodies or similar material can be seen. In the Flemming-haematoxylin material, on the other hand, the cells frequently show during these stages one or more masses of darkly staining material, sometimes more or less scattered, in other cases concentrated in a body lying near the spindle, and apparently drawing apart into two masses (compare figs. 80, 26). In slightly later stages two separate masses of similar material, composed of short, thick filaments, are seen approaching the spindle poles (fig. 27); and at full metaphase masses of the same or similar material are found lying at the spindle poles (figs. 28, 33).

From this time forward a marked and constant contrast appears between the Benda slides and those prepared by the Flemming-haematoxylin method. In the former the centrioles are conspicuously visible at the apices of the spindle at every stage (figs. 29, 34), as may be seen with perfect clearness in hundreds of cells. In the Flemming-haematoxylin slides, on the other hand, from the metaphase onward the centrioles are usually invisible, the spindle poles being occupied by a perfectly distinct irregular mass of dark-staining material which is very often drawn out along the spindle fibers for a short distance so as to give a somewhat stellate appearance (figs. 28, 30, 31). Only now and then can a distinct centriole-like body be seen in the midst of this mass. No observer who compares a hundred or more metaphases after preparation by the two respective methods can fail to be struck by the con-



spicuous difference between the two cases—a difference seen as clearly in the second mitosis as in the first (figs. 33, 34). It is obvious that the failure of the centrioles to appear in the Flemming metaphases is due to their concealment by a darkly staining material which in the Benda slides either fails to stain or has been destroyed. This difference is exactly parallel to that earlier seen in case of the idiozome complex. The conclusion thus becomes probable that the polar caps in the Flemming slides consist of the same material as the deeply staining bodies seen at an earlier stage in the idiozome complex; in other words, that those caps consist of the remains of Golgi bodies damaged by the reagents. Unfortunately, the few slides prepared by the Weigl and Kopsch methods failed to give a definite result on this point, and a decision must await further studies.

If my surmise be correct, we have before us one of the most definite types of dictyokinesis thus far made known. The nearest approach to it known to me is that described by Ludford and Gatenby ('21) in the spermatocytes of *Lymnaea* and by Nasonov ('22) in the spermatogonia of Triton; but in neither of these is the polar concentration of the Golgi bodies so extreme as is the case with material of the polar caps in *Opisthacanthus*. The latter case closely resembles that of Triton in the fact that most of the material in question remains in a single mass that is drawn out beside the spindle and there divides into two (compare my fig. 26 with Nasonov's figs. 2 to 5), though in *Opisthacanthus* some of this material apparently may remain separate (compare figs. 30, 31). It is to be hoped that this point may be tested by a suitable study of new material fixed for the Kolatchev or other suitable process.

In later stages of the first division the polar cap is found lying near the periphery in each daughter cell (fig. 31) and in the ensuing interkinesis is seen as an elongate plate-like mass lying beside the nucleus (fig. 32). These conditions are repeated in the second mitosis, so that each spermatid receives a similar mass (figs. 33, 35), but I have not been able

to trace its fate or to discover any connection between it and the acroblast. For the present, therefore, the questions here raised must remain open.

In *Centrurus* no trace of a corresponding material has been found during the divisions; but in the young spermatids a rod-shaped or plate-shaped body is occasionally seen lying near the base of the nucleus (fig. 18) or behind the nebenkern (fig. 19). This may be the remnants of an acroblast or may correspond to Gatenby's 'post-nuclear body' ('29). It would be interesting to follow out the questions here raised.

#### COMMENT

The pains taken by nature (so to speak) to ensure at least an approximately equal distribution of the chondriosomes to the sperm cells is a noteworthy fact, and one that is especially impressive in case of the *Centrurus* ring. Nevertheless, we can hardly attribute to it any fundamental importance in view of the slight but unmistakable inequality of distribution that is demonstrable in *Opisthacanthus* and seems probable in many other cases. The important part played in the construction of the sperm by the chondriosomes and Golgi bodies is none the less a fact worthy of close attention, especially on the part of those who would maintain, for instance, that "neither the Golgi bodies nor the chondriosomes are structural elements in the cellular architecture."<sup>11</sup> Without entering into the vexed questions here encountered, I will try to indicate how the singular conditions seen in *Centrurus* may in some measure be brought into relation with those existing in other forms; and I will also touch on some more general aspects of the phenomena.

Although the concentration of the entire chondrioma in a single body prior to the spermatocyte division and its ensuing exact division still remains unique in the literature of the chondriosomes, the case appears somewhat less anomalous when we consider the conditions existing in some other forms. As has been shown, the *Centrurus* ring arises by the fusion

<sup>11</sup> Tennent, Gardiner, and Smith ('31).

of a considerable number of previously separate bodies. This process is analogous to the long-familiar one shown in the formation of the nebenkern (single or double) in the spermatids of insects, but is more directly comparable to the less extensive fusion occurring in the spermatocytes in some other forms. The extent to which this process is carried seems to vary in different species. In the Lepidoptera, for example, Gatenby ('17), following out the earlier work of Meves ('00), found that the spermatocyte chondriospheres are derived from much smaller and more numerous chondriosomes of more usual type (small granules, rods, or filaments) which not only undergo subsequent increase in size, but also decrease in number by fusion as the growth period advances. At the end of this process they are still too numerous to be counted, but the final number can be seen to vary with the species. "Both *Smerinthus* and *Pieris* have a larger number than *Spilosoma*, and the latter has a larger number than *Orgyia antiqua*" (op. cit., p. 491). Their fate is substantially the same as in scorpions; the chondriome material is successively reduced during the divisions to one-half and one-fourth, the products being thereupon drawn out to form the tail sheath.

In scorpions of the *Opisthacanthus* type no fusion of the chondriosomes has yet been certainly seen; but here, too, the spermatocyte final number varies with the species. In the Indian scorpion *Palamnaeus* Gatenby and Bhattacharya ('24) found the number at the end of the growth period to be at least forty,<sup>12</sup> of which, after completion of the divisions, each spermatid receives from five to twelve. In *Euscorpis* Sokoloff ('13) found the final spermatocyte number to be twenty-four and that of the spermatids to vary from five to seven, as in *Opisthacanthus*. In *Vejovis*, as above reported, the spermatocyte number is about twenty and that of the spermatids, five, or rarely four.

<sup>12</sup> These authors describe stages of fission on the part of the spermatocyte chondriospheres during the early growth period, but in *Opisthacanthus* such a process has escaped my attention.

Beside the foregoing examples may be placed that of the snail *Paludina* and the annelid *Saccocirrus*, though the final condensation is here longer delayed. In *Paludina* (Meves, '00; Gatenby, '18) the spermatocyte chromosomes are reduced to four or five thick threads which are cut crosswise in each division and only condense into chondriospheres in the spermatids. In the annelid *Saccocirrus* (Gatenby, '22) the young spermatids contain numerous small, spherical mitochondria which later fuse progressively to produce three large chondriosomes constituting a *nebenkern*, which is later drawn out as usual to form the tail sheath.

In the light of these and similar facts, the case of *Centrurus* loses, I think, somewhat of its anomalous aspect, though it still remains an unsolved cytological puzzle. The unique features are the early period at which the condensation occurs, the extreme degree to which it is carried, the remarkable form of the product, and, above all, the accuracy of its division and distribution to the sperm cells. The *Centrurus* type agrees, however, with the *Opisthacanthus* in that early in the growth period all the chondriosomes are closely massed around the idiozome. The subsequent differences arise from the fact that in the one case the chondriosomes soon part company, scatter through the cytosome, and remain permanently separate, while in the other they remain in situ and fuse permanently to form a single body. What determines this fusion cannot now be stated; but the resulting ring shape is at least partially conditioned by the circumstance that the fusing chondriosomes are massed in the restricted space that surrounds the cell axis between the idiozome and the nucleus; hence also the fact that the completed ring always surrounds the axis, with the idiozome lying just below its center.

All this seems fairly simple. More interesting, and far more difficult, is the problem offered by the regular division of the ring and the equal bipolar distribution of the products. This question cannot be separated from those relating to the sorting out of whole chondriosomes (as in *Opisthacanthus*) and the segregation of the more usual type of these bodies

during mitosis (e.g., in the insects). In *Opisthacanthus* the distribution of the chondriosomes is approximately equal, but not exactly. All points to the conclusion that the process is here one of random assortment; before the division the chondriospheres show an apparently haphazard grouping, and since both divisions are equal, chance should favor a nearly equal distribution to the products.

In the more usual case, where the chondriosomes have the form of threads or chondriocoats, the case is more complicated, involving a mixed process of division and random assortment (e.g., in many insects). Here the threads, after certain preliminary movements, lie during the anaphases parallel to the spindle, closely investing it to form a mitochondrial sheath or palisade.<sup>13</sup> In the course of the division as is shown with great clearness in Bowen's figures of hemipteran spermatocytes, those chondriosomes which happen to lie in or near the polar regions pass undivided into the daughter cells, while those in the equatorial regions are cut in two, equally or unequally, as the cell constriction deepens. There is little here to suggest an autonomous act of division, comparable to that of the chromosomes. On its face the division offers the appearance of a merely mechanical result of the cell constriction; and Bowen considered it 'practically certain' that such is actually the case.<sup>14</sup> Bělař seems to favor the same view ('26, p. 381), which is also not inconsistent with many figures in the literature.

Nevertheless, the evidence for this view seems to me inconclusive, especially in view of the facts in *Centrurus*, which can be more closely studied than in most other known cases. At first sight those facts may seem to point to an autonomous act of division on the part of the chondriosome ring; but I

<sup>13</sup> See, for instance, the figures of Giglio-Tos ('08), Lewis and Robertson ('16), and Bělař ('27-'29) for Orthoptera; of Duesberg ('10) for Coleoptera; and of Bowen ('20-'22) for Hemiptera.

<sup>14</sup> "The mitochondrial threads which happen to lie in the path of the constriction are thus at last caught between the spindle and the cell wall and seem to be severed mechanically into two parts," etc. ('20, p. 325). See also Bowen, '27, p. 317.

find it difficult to adopt such an interpretation, in view of the phenomena seen in other types of chondriokinesis. In many respects the ring offers during its division the aspect of a plastic body that is passively pulled in two by forces acting in opposite directions between the equator of the spindle and the poles. The initial effect (to repeat) is a drawing out of the circular ring to an elliptical form during the general karyokinetic elongation (figs. 7, 8), and this is followed by its polar breakage to form two half-rings which soon assume the form of two parallel rods (figs. 7, 8). Later these are divided across the middle point (fig. 9), the products moving well apart at a time when the cell constriction is still far from completed (fig. 10).<sup>15</sup> Similar appearances are seen, with at least equal clearness, during the second mitosis. Here, as earlier indicated, the two chondriosome rods are often slightly constricted, and show a lighter middle zone, before the slightest sign of a cell constriction has appeared (fig. 14). A little later these rods, as in the first division, are seen drawing apart into two halves some time before the cell constriction is completed (figs. 15, 16).

The foregoing considerations, admittedly, do not wholly exclude the occurrence either of an autonomous division of the ring or of its mechanical bisection by the cell constriction; nevertheless, I think the question should be kept open by the consideration of other possibilities. The division of the *Centrurus* ring recalls in some respects that of the nucleolus and the ensuing migration of the products to opposite poles—a process that occurs during mitosis in some of the Protista and sometimes in higher plants.<sup>16</sup> In *Cucurbita* this process, though of exceptional occurrence, is especially interesting because the division and poleward migration of the products are completed while the chromosomes are still in metaphase and before they have begun to move. Inasmuch as the nucleolus lies wholly within the spindle area, Frew and Bowen conclude that:

<sup>15</sup> Compare Bowen's somewhat similar figure 17 of the corresponding division in *Euschistus* ('20).

<sup>16</sup> See the interesting account of this phenomenon in *Cucurbita* by Frew and Bowen ('30), with a review of the literature.

The spindle represents a region in which are localized those forces, of whatever nature, that are responsible for anaphasic movements. . . . The nucleolus may be thought of in terms of some foreign body inserted into the spindle-area . . . [and] presumably any other small mass of proper consistency inserted into the spindle would behave in the same way (op. cit., p. 208).

We may briefly consider some of the questions thus raised, though none of them can yet be answered with certainty. The conclusion just cited cannot apply, obviously, to division of the chondriosomes; for all observers are agreed that these bodies are never found within the spindle area. This fact appears with perfect clearness in *Centrurus*, the ring lying quite outside the spindle and sometimes almost at the cell periphery. Nevertheless, the possibility remains that its division (and perhaps that of other forms of chondriosomes) may be caused by cytoplasmic activities in the region lying outside the spindle area. We have long been familiar with the fact that mitosis, sometimes in its earlier stages, before establishment of the spindle, is commonly accompanied by linear orientations, alignments, or movements on the part of various formed bodies, lying outside the spindle. In their fullest development these orientations are found to follow curved lines focused toward the spindle poles.<sup>17</sup> The bodies in question may include not alone the chondriosomes and Golgi bodies (as in chondriokinesis or dictyokinesis), but various others, including yolk, pigment, secretory granules, microsomes, and perhaps plastids. These phenomena show (to cite a recent statement) that "the bipolarity that has long been evident with respect to the chromosomes, spindle and asters is also clearly shown by the orientation of cytoplasmic components."<sup>18</sup> Numerous observations, both on living and fixed cells, long ago made it evident that yolk granules, microsomes, pigment, and the like are aligned radially between the astral rays during mitosis—this is, in fact, the only visible evidence of the existence of the asters in the living object.<sup>19</sup> Since the

<sup>17</sup> See, for example, O. Hertwig ('90), Brauer ('93), Meves ('07), Wilke ('13), Bowen ('20), Pollister ('30), and many others.

<sup>18</sup> Pollister, op. cit.

<sup>19</sup> Compare Wilson, "The Cell," pp. 142 ff.

lines of orientation in question lead toward the astral centers, we can hardly escape the conclusion that they should be regarded as continuations of the peripheral regions of the asters (i.e., of the interradii), and hence as belonging to the general mitotic field.<sup>20</sup> Their nature and causation therefore involve the same problems as those offered by the astral rays in the narrower sense; but it must be admitted that those problems still remain in large degree unsolved.

The old notion of the astral foci (or central bodies) as centers of attraction is not yet wholly extinct, nor is Boveri's conception of the central bodies as 'dynamic centers' in which (to cite the words of a later writer) "are centered at the time of division influences that extend to practically all the elements of the cell and direct their division in an orderly manner."<sup>21</sup> Such a conception must, however, reckon with the disconcerting phenomena of anastral mitosis in which neither asters nor central bodies are present; yet in some cases, it would seem, certain bipolar orientations or movements of the cytoplasmic formed bodies take place that are similar in type, if less conspicuously shown, to those seen in amphiastral mitosis.<sup>22</sup> This fact is emphasized by Bowen in a subsequent paper. "The plant seems to achieve without centriole or aster exactly those cytoplasmic arrangements for the appearance of which in animal cells appeal is generally made to the aster and its center"<sup>23</sup> (Bowen, '29, p. 151). This is perhaps somewhat of an overstatement; but obviously a serious difficulty is here indicated, and one which leaves our views concerning cytokinesis in a very confused state.

<sup>20</sup> This would harmonize with Conklin's early suggestion ('02, '31) that "the asters are chiefly concerned with intra-cellular movements and the localization and division of the materials of the cell-body." I have long suspected that this assumption may explain the strange fact that in fertilization the sperm asters often undergo an extreme development, only to become much reduced, or even to disappear completely, before the definitive cleavage asters are developed. For a striking example of this see Coe ('99) on the nemertine egg.

<sup>21</sup> Bowen, '20, p. 348.

<sup>22</sup> See Nassonov ('18), Bowen ('29).

<sup>23</sup> See especially Spek ('18), Bělař ('27, '28, '29). Review in Wilson, '25, pp. 192 ff.



Without entering far into the complicated problems here at issue, I will indicate the more promising outlook that seems to be opened in this field by researches on the protoplasmic currents that accompany mitotic cell division.<sup>23</sup> Since the early work of Fol and others in the seventies it has been known that the astral rays are the seat of centripetal currents of hyaloplasm flowing toward the astral centers, while the granules of the interradii move in the opposite direction. These currents are associated with, if not directly a part of, the general system of vortical cytoplasmic currents that accompany mitosis, made known by the early work of Bütschli and of Erlanger and later investigated with so much success especially by McClendon and by Spek.<sup>24</sup> These currents, as is well known, flow superficially from the region of the mitotic poles toward the equator, thence inward, and finally poleward along the spindle. The possibility is here suggested that the bipolar orientation and alignment of cytoplasmic formed bodies outside the spindle may be determined by such currents, and even that the same cause might in some cases determine their division.

As I have reviewed in another place,<sup>25</sup> several of the earlier observers recorded the fact that under the influence of such currents various formed bodies (granules, pigment) tend during mitosis to accumulate in the equatorial region and to pass thence inward toward the spindle; and it was later found that the chondriosomes show a similar behavior.<sup>26</sup> Bělař, in particular, referred the formation of the 'mitochondrial palisade,' which surrounds the spindle during the anaphases, to the fact that these bodies, after moving inward, are carried along the spindle toward the poles in opposite directions by the axial currents. The pregnant suggestion is added that if poleward currents can thus cause the division and bipolar movements of an aggregate of granules or other

<sup>23</sup> See footnote, page 459.

<sup>24</sup> See "The Cell," 1925, pp. 194 ff.

<sup>25</sup> Op. cit., p. 195.

<sup>26</sup> See especially Bělař, '27, '28 (figs. 137, 138), '29. Also the critical review of Wassermann, '26.

small separate bodies, they might likewise be responsible for the division of larger plastic bodies that lie in the corresponding position.<sup>27</sup> This suggestion is offered, no doubt, in only a tentative spirit, yet it is one that seems worthy of serious consideration as a possible clue to the division of the *Centrurus* ring. The 'currents' in question are very slow movements or lines of strain in the viscous protoplasm surrounding the spindle. It seems quite possible that such an agency may play some part in the preliminary elongation of the ring and may even create a bipolar traction that is responsible for its subsequent division. Light on this question may perhaps be found by experiments (e.g., by centrifuging) on the living material. For the present, however, the foregoing suggestions are hardly more than crude hints having little value save as possible incentives to further study of an interesting problem in cell mechanics.

Such uncertainties emphasize the meagerness and vagueness of our present knowledge concerning the cell activities during mitosis which so impress the cytologist. The same situation is even more striking in respect to the plastic and formative activities of the cell. Here, too, the fact is not to be disguised that in spite of many ingenious and beautiful researches our understanding of the mechanism of development and differentiation still remains in high degree rudimentary. Chief among the disconcerting puzzles that here confront us is the uncanny ability of the organism, or of nearly related organisms, to achieve the same results by widely different methods. A thousand examples of this, many of them far more remarkable than that shown by the sperm formation in scorpions, might be cited from the literature alike of cytology and embryology. The case of *Opisthacanthus* and *Centrurus* seems a simple matter when compared (to take another cytological example) with the amazing vagaries dis-

<sup>27</sup> "Denn es liegt auf der Hand dass was bei der Zellteilung mit einem Aggregat von Körnchen geschehen kann . . . . unter Umständen auch mit einem leichtflüssigen grösseren Gebilde, welches an derselben Stelle liegt, geschehen kann" (Bělař, '28, p. 94).

played during the sperm formation in the manoeuvres of the chromosomes in *Protortonia* (a coccid hemipteran) in contrast to those that we are accustomed to regard as 'typical' in other insects, yet the final result seems to be identical (F. Schrader, '31). In the field of embryology a long-standing puzzle is the formation of a typical embryo in the ascidian or bryozoan by two such widely different processes as appear in the egg development and in the bud development. Not less notorious is the startling contrast between the formation of the lens in the eye of the larval salamander by regeneration after its experimental extirpation and the same operation as performed during the original normal development of the egg. In the same light we may regard those remarkable modifications of development produced by 'organizers,' a field of inquiry which in the hands of Spemann and his followers has come to occupy a position of central interest in the embryological research of our time. Superficially viewed, each of these operations looks simple enough; but who will have the hardihood to maintain that any one of them lies within the range of our present comprehension?<sup>28</sup>

In this respect the operations of sperm formation offer an aspect quite analogous to that seen in the early development of the egg. In both cases we possess a certain amount of knowledge concerning the basic protoplasmic activities that underlie the visible performance of the cell. In both we have a fairly adequate acquaintance with the superficial aspects of the phenomena. To a limited extent we are able to follow them with the eye. With the aid of the microscope we catch glimpses of the preparation and movements of building materials, of their carefully ordered assembly and molding to produce a specific type of form and structure. Yet the

<sup>28</sup> Compare J. Gray. "It seems more rational to regard living material as a state of matter where the constituent molecules are organized in a way quite unknown in the inanimate world, and which will only be elucidated by methods of analysis which have yet to be discovered. . . . Until we have the means to explain the situation in much greater detail, a knowledge of our ignorance is perhaps the most valuable asset we can hope to possess." "Experimental Cytology," p. 97, 1931, Macmillan Co.

spectacle of the cell at work is one that we view with a wonder that bears witness to our ignorance concerning what lies behind the show. It is as if the performance were carried through with perfect understanding and competence, but with no intention of explaining the trick. It is a tantalizing but profoundly interesting display and one that places before our eyes an alluring field for further exploration by experimental research.

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## PLATES

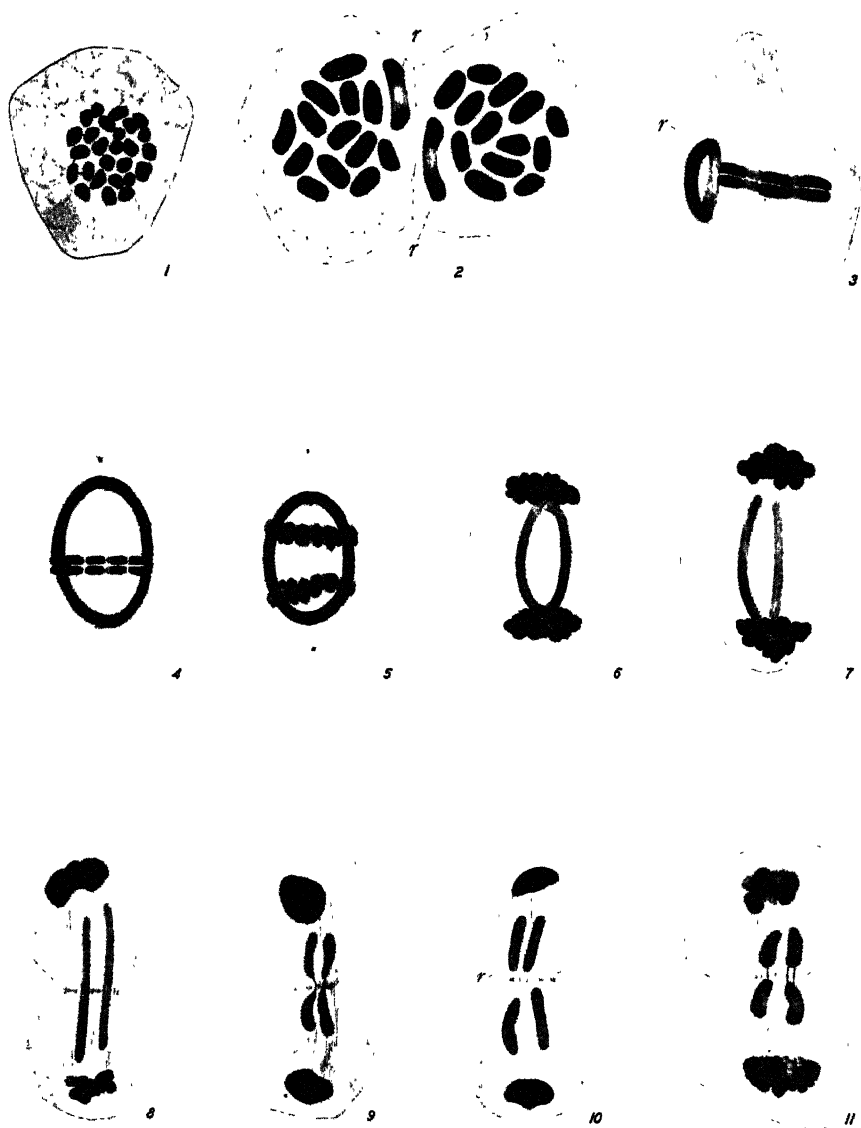
### EXPLANATION OF FIGURES

All the figures were outlined with the camera lucida (Zeiss 1.5 apochromatic, oil-immersion, oc. 12, tube 160) and are here reproduced at an enlargement of about 225 diameters. With a single exception (fig. 25), they are in no manner schematized except that in a few cases (e.g., in figs. 28, 29) some of the chromosomes or chondriosomes have been omitted for the sake of clearness. About two-thirds of the figures were drawn by the late Mabel T. Hedge, whose conscientious accuracy was well known, and the remainder by the author. The fact may be emphasized that in many of the figures (e.g., figs. 28 to 31, 34, 49 to 51) the chondriosomes are shown superposed on the spindle area, giving the erroneous impression that they lie within the latter. The true relations are shown in figure 48.

The methods most commonly employed are designated in the descriptions as F.H. (strong Flemming, iron-haematoxylin), B. (Benda fixation and stain, i.e., alizarin-crystal violet), and B.H. (Benda fixation, iron-haematoxylin). Other abbreviations are:

*a*, acrosome  
*c*, chondriosomes  
*i*, idiozome

*p*, polar masses, perhaps Golgi material  
*n*, nucleus  
*r*, chondriosome ring or its products



## PLATE 1

### EXPLANATION OF FIGURES

*Centrurus exilicauda* (F.H.). First spermatocyte division.

- 1 Spermatogonial metaphase, twenty-six chromosomes.
- 2 Two adjoining first spermatocyte metaphases, thirteen chromosomes plus the chondriosome ring (*r*) in axial view.
- 3 Similar metaphase in side view, with ring.
- 4 The same, with ring in face view, karyokinetic elongation.
- 5 and 6 Two successive anaphases; elongation of the ring.
- 7 Final anaphase. Polar breakage of the elongated ring.
- 8 to 10 Three successive views of telophases; transverse division of the chondriosome rods.
- 11 Final telophase, cleavage completed.



## PLATE 2

### EXPLANATION OF FIGURES

*Centrurus exilicauda* (F.H.). Second spermatocyte division. Sperm formation.

12 Interkinesis, with the massed chromosomes and the two chondriosome rods ( $r$ ).

13 Second metaphase, primary elongation of chondriosome rods.

14 Final anaphase, shortening of chondriosome rods.

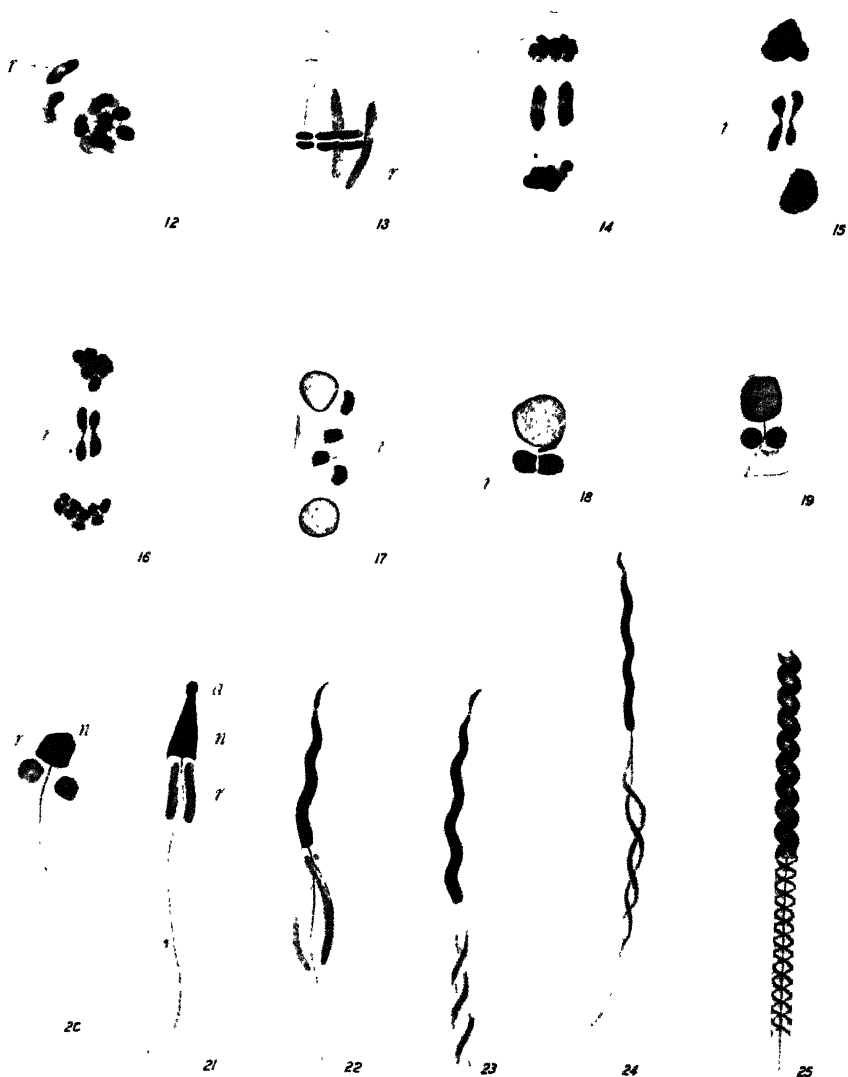
15 and 16 Division of the chondriosome rods ( $r$ ).

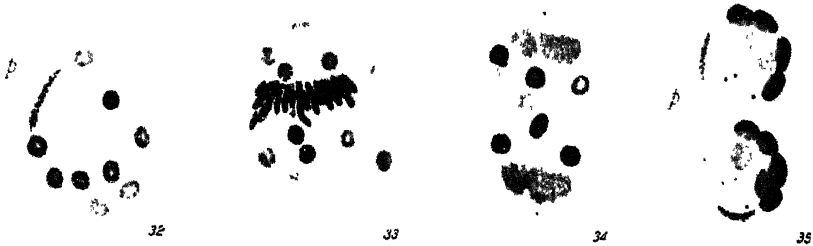
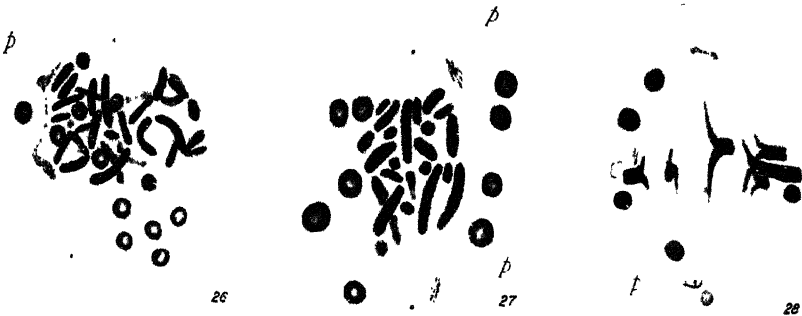
17 Final telophase.

18 and 19 Early spermatids, successive stages with double nebenkern and possible Golgi material.

20 to 24 Successive stages of elongation and formation of the tail envelope.

25 Diagram of advanced stage of the elongation; twisting of the nucleus probably a result of contraction.





### PLATE 3

#### EXPLANATION OF FIGURES

*Opisthacanthus elatus*. The spermatocyte divisions.

- 26 and 27 Two successive stages of first division, showing chromosomes, central bodies, and polar masses (*p*), perhaps Golgi material (F.H.).
- 28 Metaphase, with dividing chromosomes and polar caps (F.H.).
- 29 Similar stage showing centrioles and polar granules in the chromosomes (B.).
- 30 Late metaphase, polar caps (F.H.).
- 31 Telophase (F.H.).
- 32 Interkinesis. Polar material (F.H.).
- 33 Second metaphase, polar caps (F.H.).
- 34 Second telophase (B.).
- 36 Spermatids, nucleus, six chondriospheres, polar material (F.H.).

## PLATE 4

### EXPLANATION OF FIGURES

*Opisthacanthus elatus*. Sperm formation (B.).

36 and 37 Young spermatids, nucleus, chondriospheres, acrosome, blepharoplast (or the like), axial filament.

38 to 40 Formation of chondriosphere ring, stages of elongation.

41, a, b, c The three observed types of chondriosphere ring, in axial view.

42 to 44 Later stages of elongation.

45 and 46, a Three successive stages of backward drawing out of the chondriospheres, to form the tail envelope. The dark body near the base of the nucleus in figures 40 to 44, 46, 46, a, is perhaps the 'post-nuclear body' of Gatenby.



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37



38



39



40



41



b



c



44



42



43



45



46



46a



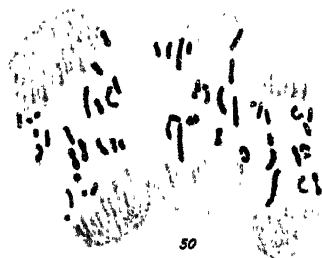
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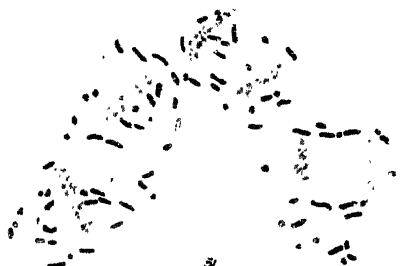
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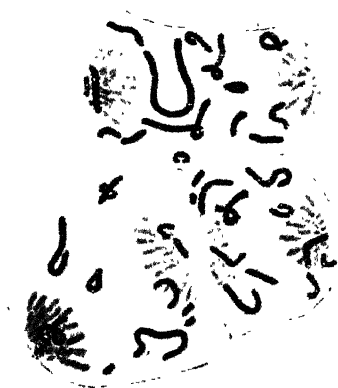
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## PLATE 5

### EXPLANATION OF FIGURES

#### The spermatogonia (B.).

- 47 *Opisthacanthus*, early spermatogonial cyst.
- 48 Probably early spermatogonial mitosis.
- 49 *Vejovis boreus*, spermatogonial mitoses, small type of chondriosomes, some fragmenting.
- 50 *Opisthacanthus*, type similar to last.
- 51 *Centrurus exilicauda*, similar type.
- 52 *Opisthacanthus*, large type, chondriosomes fragmenting, cell boundaries not visible.
- 53 The same, anaphases, probably of same type as the last. Sorting out of whole chondriosomes.



## PLATE 6

### EXPLANATION OF FIGURES

Centrurus. Spermatocytes, early and late growth periods (figs. 54 to 59, 63, a to k, from *C. henzi*, the others from *C. exilicauda*).

54 to 59 Young polarized stages with chondriosomes massed at one pole (B.).

58 Two polar views of similar stage to show chondriosome group (B.).

60 Polarized stage in *Opisthacanthus*, for comparison (B.).

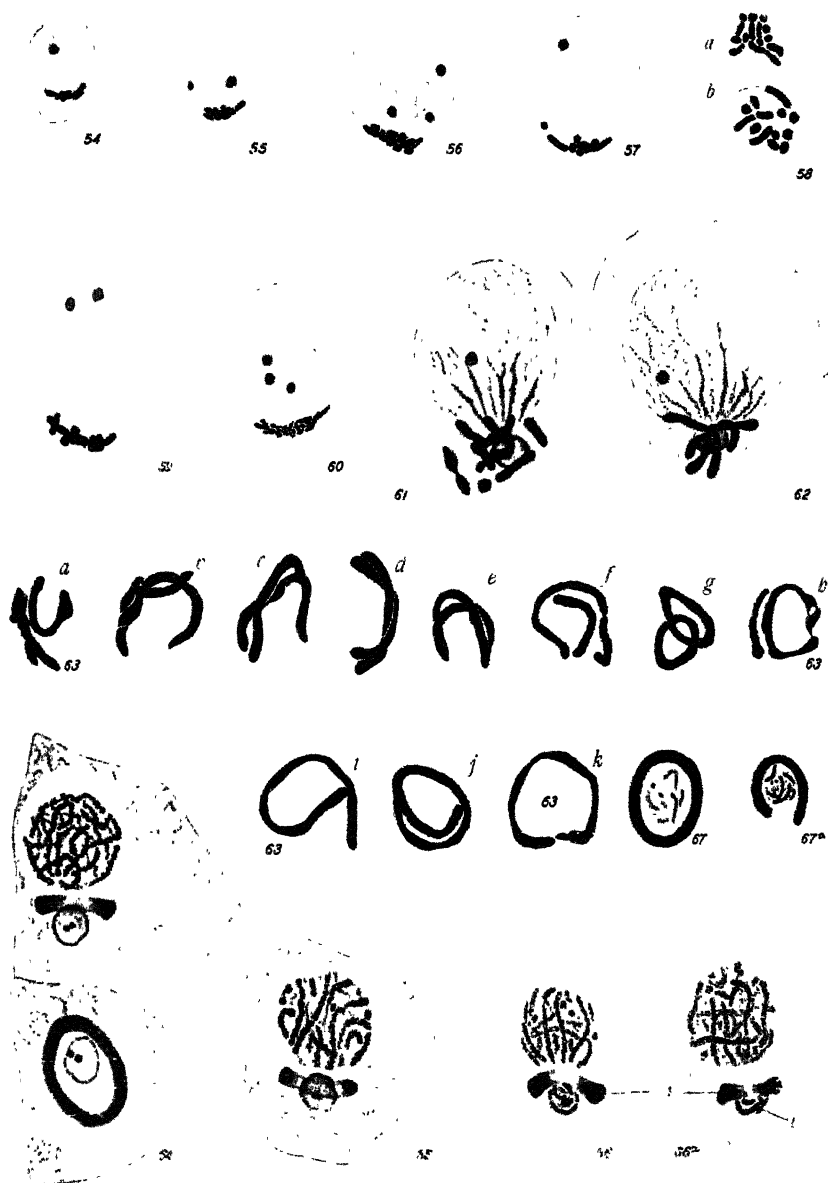
61 and 62 Bouquet and amphitene stage (*exilicauda*). From a Benda-Bensley preparation.

63, a to k Stages in the ring formation (F.H.).

64 and 65 The completed ring, spheroid type of idiozome (F.H.).

66 and 66, a Side view of ring and idiozome. Golgi bodies (?) (B.H.).

67 and 67, a Axial views of stage like last (B.H.).



## PLATE 7

### EXPLANATION OF FIGURES

Opisthacanthus. Growth period and early prophase.

68 and 69 Early spermatocytes, polarized (compare fig. 60), early stage of scattering of the chondriosomes (B.H.).

70 Later stage, chondriosomes scattered, exposing idiozome (B.H.).

71 Conversion of the rods into chondriospheres. Idiozomes (F.H.).

72 Early middle growth period, axial view. Idiozome (B.).

73 Nuclear polarized stage, side view, idiozome (B.).

74 to 77 Later stages, side view. Idiozome (F.H.).

78 Polarized nucleus, polar massing of chondriospheres.

79 and 80 Early prophase, showing Golgi material (?). Late synizesis (perhaps due to fixation) (F.H.).





# THE ONTOGENETIC HISTORY OF THE MITOCHONDRIA OF THE HEPATIC CELL OF THE WHITE RAT

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ONE TEXT FIGURE AND ONE PLATE (TWENTY-FIVE FIGURES)

## AUTHOR'S ABSTRACT

This work was designed to determine the ontogenetic variability of the morphology of mitochondria of the hepatic cell.

The mitochondria of the cells of the hepatic diverticulum are minute spheres. These spheres enlarge, become associated, forming beaded filaments, which in turn become smooth filaments, the process culminating in the twenty-day fetus. There is no individual or lobular variability in fetuses of a given length. One-half day before birth the above process is reversed, so that separate spheres are found in the eight- to twelve-hour young. This enlargement and enspherulation are accompanied by an accumulation of large quantities of glycogen. After this age, the spheres become associated into beaded filaments again and the glycogen disappears, the cycle ending at thirty-three hours. From two to eight days of age there is great individual variability.

Mitochondrial morphology characteristic of the adult is first found in the fourteen-day young, when cells contain long, smooth or beaded filaments, rods, and spheres. There is no lobular variation as to types of mitochondria, but only in numbers, the cells around the central vein containing fewer mitochondria than other cells of the lobule. Variation between individuals after birth is due to something other than age, type of food, period of digestion, and length of starvation. Fat is formed within spherical chondriosomes.

In the first studies of mitochondria, cytologists did not take into account the possible morphological variation due to changes in the physiological state of the cell. Later, attempts were made to control such states, but heretofore no one has thought it necessary to check both physiology and ontogenetic history of the tissue in performing investigations on mitochondria. A few cytologists, Chaves ('20), Arima ('27), and Noël and Pigeaud ('30), have described the morphological changes of mitochondria in the developing hepatic cell of the hedgehog, mouse and man, and man, respectively. In each case the material investigated was inadequate both as to stages used and as to checks employed.

Noël ('23) and Okushi ('28) have demonstrated that the morphology of mitochondria of the hepatic cell varies with the stage of digestion. Consequently, if an attempt to study the ontogenetic changes of mitochondria is made without checking digestion, the picture represented would be, it is true, developmental, but with possible inaccuracies induced by varying physiological states. This consideration makes it impossible for one to safely evaluate the three brief accounts of the ontogeny of mitochondria of the hepatic cell.

In view of the fact that a complete study of the ontogeny of the mitochondria of the liver has not been made, it seems highly desirable to do so, with a view to determining whether or not the ontogenetic stage should be considered in making studies of mitochondria. In addition, if the known causes of mitochondrial changes were kept as near constant as possible in all individuals, and if the number, distribution, and configuration of mitochondria were found to be specific for a given age of embryo, it would seem logical that a study of the ontogenetic history of these cytoplasmic inclusions should, if correlated with our knowledge of the function of the organs involved, throw some light on the general problem of the function of mitochondria. Also, if constancy is found, it would seem likely that some of the permeability phenomena of the placenta might be studied by administering treatments known to alter the morphology of the mitochondria of the maternal liver and observing the effect on the fetal liver.

#### MATERIAL AND METHODS

The albino rat, *Mus norvegicus albinus*, was selected as material because of its ready availability to all workers and its common usage in laboratory work of all kinds. The pregnant females were killed by decapitation twenty-four hours after they had been fed. The fetuses were measured and the livers of both fetuses and mother were excised and fixed.

The following fetal material was used:

<i>Mean length in mm.</i>	<i>Approximate age in days, according to Stotsenburg ('15)</i>	<i>Number of litters</i>
3.0		1
4.0		1
4.5		2
7		1
8	13.6	2
11.75	15	1
13.5	16	1
14	16.2	1
16.5	17	1
22	19	1
28	20	1
30.1	20.2	1
32.5	20.5	2

In all cases at least two individual livers were examined from each litter. This was regarded as adequate material, since absolutely no individual variability was found. The remaining livers were embedded in paraffin and kept, in case later experience should suggest their use.

The account of mitochondrial behavior after birth is based on the following material:

<i>Age</i>	<i>Number of livers studied</i>	<i>Number of litters represented</i>
At delivery	2	2
$\frac{1}{2}$ hour	1	1
1 hour	1	1
$1\frac{1}{2}$ hours	1	1
3 hours	2	1
4 hours	1	1
8 hours	2	2
12 hours	2	2
15 hours	1	1
18 hours	2	1
24 hours	1	1
33 hours	2	2
48 hours	2	2
78 hours	2	2
5 days 10 hours	2	2
6 days 2 hours	2	1
7 days	2	2
8 days 6 hours	2	2
12 days 4 hours	2	2
14 days 10 hours	2	2
19 days	2	1
23 days	2	2
26 days	1	1
29 days	1	1
35 days	2	1
50 days	1	1



No exact check was possible on the length of time between the last feeding and delivery, but all young used were born between fourteen and eighteen hours after the mother had been fed. Young over one day old were taken from mothers twenty-four hours before killing, and those twenty-four hours old or less were taken at the time of delivery, thus preventing nursing. All were killed by decapitation.

For adult material I have examined livers from the following: nine pregnant females, three non-pregnant females, and three males. All were killed by decapitation twenty-four hours after the last time of eating. It might be well to note that the colony was fed once each day and that on the last day of feeding, before killing, they were permitted to eat all that they would in one hour and the remainder of the food, if any, was removed so as to avoid the possibility of animals being studied during digestive activity.

The tissue was fixed and stained according to methods of Regaud, Benda, and Cowdry's modification of Altmann. Since better and surer results were obtained by the use of Regaud's method, it was used in the majority of cases. Sections were cut at  $4\mu$ .

For the study of prepared sections a Leitz binocular with apochromatic equipment was used for the most part. In order to make accurate comparison of mitochondrial size at various ages, four microscopes were set up; then, by changing only two at a time, it was possible to make a careful evaluation of the relative sizes. This method afforded a very good check on camera-lucida sketches.

Temporary preparations were utilized for the determination of glycogen. Immediately upon excision, the livers were teased in absolute alcohol saturated with iodine, to be examined a few minutes later. This method saves a great deal of time and can be made very accurate by always fixing additional pieces of tissue in pure alcohol, immersing in water, and then staining with iodine. Best's carmine method was also used.

## OBSERVATIONS

*3-mm. embryo to 14-mm. fetus*

The cells of the hepatic diverticulum of the 3-mm. embryo have extremely dense cytoplasm, that is, the cytoplasm must have contained a large amount of coagulable protein that takes and holds stain. Dark strands of the ground cytoplasm extend from the cell wall next to the primordial bile duct to the nucleus, and are approximately parallel with each other. The cytoplasm of the distal portion of the cell contains strands which lie in no definite order. These cells possess very small spherical mitochondria, barely in the realm of microscopic visibility, which tend to lie in the darkly staining cytoplasmic strands (fig. 2). It is to be noted that these mitochondria are exactly like those found in the cells of the gut wall.

In the 4- and 4.5-mm. embryo, the endodermal cells which have migrated into the septum transversum and the cells which will become the ductus choledochus have the same type of mitochondria. The spheres are very little larger than in the 3-mm. embryo, and are located in the densely staining strands which are scattered at random.

The larger and more irregularly shaped hepatic cells, frequently in the form of truncated pyramids, of the 8-mm. embryo have a more or less homogeneous and very dense cytoplasm. As regards the mitochondria, a few short rods are formed, but regular spheres about twice the size of those of the cells of the hepatic diverticulum are in predominance. Short, beaded filaments are formed by the association of two or three, sometimes four, spheres, held together by a fine thread which exhibits the same staining reaction as the spheres. The two possibilities would, of course, come to mind, either that these are spheres in the process of dividing to form more spheres or that they might be separate spheres coming together to form a beaded filament. If the former were true, one would expect to find various stages in the division process, but such is not the case. Although the strands connecting the spheres are somewhat variable in length and diameter, they are always relatively tenuous, with

their length nearly equal to the diameter of the spheres. In addition, as the beaded filaments are formed the number of mitochondria is decreased, which would not suggest that the connected spheres are in process of division. Thus it would seem that in the 8-mm. embryo the mitochondria of the hepatic cell are exhibiting the initial steps in the transformation of spherical mitochondria into beaded filaments and that the procedure followed is the association of spheres already present (fig. 3). When these cells were first examined by the use of an ordinary table lamp, the filaments appeared smooth, but later, by the use of stronger light, a 4-ampere arc light, the filaments appeared distinctly beaded. Probably beaded filaments would be observed more frequently in mitochondrial studies if stronger light were used.

The hepatic cells of the 11.75- to 14-mm. fetuses are much the same shape and size and have almost as dense cytoplasm as those of the 8-mm. embryo. The mitochondria are about one and one-half times as large. The process of filament formation by the association of spheres has continued, so that more spheres are held together by tenuous strands, leaving fewer free spheres. In the 11.75-mm. fetus the free spheres are the same shape and size as those in the filament, resembling the 8-mm. in that respect. Those of the 13.5-mm. and 14-mm. tend to be more oval than round and the thread which holds them together is thicker. A few rods are also found at this age (figs. 4 and 5).

#### *16.5- to 28-mm. fetuses*

There is a marked increase in the size of the hepatic cells of these fetuses over those previously described, as is seen in figures 6, 7, and 8. The cell shown in figure 8 is one of the smaller ones of the lobule, the average being approximately the same size as figure 7. The cytoplasm of the hepatic cells of the 16- to 28-mm. fetuses is much less dense than that described above, and is much less homogeneous, with very small irregular vacuoles in it. These vacuoles probably contained glycogen before fixation, since the similarly shaped,

though larger, vacuoles found immediately after birth do contain that substance.

In the 16.5-mm. fetus the beads of the filaments continue to elongate into ovals and the threads continue to become thickened. Also, the beads are slightly larger than those of the 13.5-mm. fetus. The process continues through the 22-mm. fetus, so that a few long, more or less even filaments result, although most of those formed still retain the appearance of ovals held together by thick siderophilic threads. These two types of filaments vary in size. The latter are larger and thicker, while those of the former type tend to be slightly thinner and much longer than those of the 16.5-mm. fetus.

This process of filament formation by the association and elongation of spheres culminates in the 28-mm. fetus (one day before birth), when only very few free spheres, perhaps one or two in each cell, are observed, and most of the mitochondria are in the form of long, even filaments, although a few beaded ones may be seen. Mitochondria at this age are approximately three-fourths the width of those found in the 22-mm. fetus.

In the 16-mm. fetus one finds, for the first time in the ontogenesis of the hepatic cell, mitochondria in the form of hollow spheres (grains à coque of Noël) which are somewhat variable in size, though usually larger than the other spheres (fig. 6). These result from the accumulation of non-siderophilic material in the center of the earlier spheres. There are relatively few hollow spheres present, usually only three or four being visible in a cell, in  $4\mu$  sections. They are approximately the same size and number in the 22- and 28-mm. fetuses (figs. 7 and 8). It is to be noted that these spheres are entirely different from those described by Kater ('31) in the hepatic cell of the cat, as the latter appeared hollow only after Benda's stain and solid after Regaud's.

In summarizing the material investigated from the time the hepatic diverticulum is formed, 3-mm., up to the day before birth, 28-mm. fetus, the hepatic cell changes from

a small, more or less regularly shaped cell with a very dense, homogeneous cytoplasm to a large, irregular-shaped, and slightly vacuolated one. The mitochondria, starting as minute spheres, enlarge and become associated with each other to form beaded filaments. The majority of these become long, even filaments by the time the fetus has reached a length of 28 mm. This process is accompanied by a reduction of the number of mitochondria. Some spheres enlarge and become hollow in the 16.5-mm. fetus and are found throughout the remainder of fetal life. A few rods are also found during these ages. The mitochondria are distributed evenly throughout the developing lobule. All individuals of the same or different litters of a given length of fetus have the same size, shape, and distribution of chondriosomes. It is of interest, I believe, that the picture of the ontogenetic changes of the mitochondria of the hepatic cell is not a non-coherent, disorganized one, but rather one of a gradual, organized, regular change, with no periods of regression. Thus, the formation of smooth filamentous mitochondria in the 28-mm. fetus is the culmination of a process of fusion of spheres that started shortly after the appearance of the hepatic diverticulum and gradually progressed toward its objective, without wavering even slightly.

*32.5-mm. fetus (one-half day before birth)*

The hepatic cells of this size fetus resemble those of the last-described group in shape and size and in character of the ground cytoplasm, except for an enlargement of the vacuoles. On the other hand, there is a marked change of the mitochondria, both as to type and size. Although filaments predominate, they are much shorter than in the 28-mm. fetus and most of them are distinctly beaded. More free and hollow spheres and rods are found. All types of mitochondria are somewhat larger than those found in the 28-mm. fetus. The mitochondrial picture of the hepatic cell at this age does not vary with individuals, just as in the younger fetuses (fig. 9).

*Birth to thirty-three hours*

The hepatic cells at birth are larger than those just described. There is a constant increase of cell size up to the eight- to twelve-hour young. After this age a reduction in size takes place, until at eighteen to twenty-four hours the hepatic cells approximate those of the 32.5-mm. fetus. This variation in cell size is apparently due to an increase followed by a decrease in volume of the irregularly shaped vacuoles. These vacuoles are formed as a result of the solution of glycogen by the aqueous fixatives used, as glycogen determinations showed bodies of this substance of a size comparable to the vacuoles. Although the livers of the 16- to 32.5-mm. fetuses were not stained for glycogen, it would seem likely that the contents of the vacuoles found in their hepatic cells is likewise glycogen. This is supported not only by the characteristic irregular outline of the vacuoles, but also by the unlikelihood of such large quantities of glycogen appearing suddenly at birth. Although the lobule usually contains cells of more or less equal size, occasionally those around the portal unit are smaller and tend to be non-vacuolated. Thus glycogen first disappears from the cells bordering the portal vein. There is a wide variation in the size of the hepatic cells and in the size of the vacuoles in young of the same age, even though they are litter mates (figs. 12 and 13, 16 and 17). This seems to indicate that the glycogen is accumulated in amount and utilized or absorbed at a rate which varies with individuals. The ground cytoplasm remains about the same throughout this period.

The change in mitochondrial form which started in the 32.5-mm. fetus—that is, the formation of beaded filaments from long, even ones and the breaking off of these beads to form free spheres—has continued in the hepatic cell at birth, so that there are fewer and shorter smooth and beaded filaments and more free spheres. This process of formation of free spheres from beaded filaments tends to culminate in the eight-hour hepatic cell (fig. 12), when the spheres are much in predominance, although a few of the beaded filaments

remain. After the eight-hour stage, the spheres become associated, forming beaded filaments again (figs. 13, 16, and 17), so that at eighteen hours after birth that type of chondriosome is in the majority. This is accompanied by a reduction of the size of the previously described glycogen vacuoles. The data obtained seem to indicate that, although this cycle takes place in all individual hepatic cells between parturition and thirty-three hours after, young of the same age may be in different phase of the cycle (figs. 12 and 13, 16 and 17). Hollow spheres remain throughout this period, and some of them become very much larger than they were during fetal life.

Because of the above-described process, the number of mitochondria starts to increase just before birth and continues to increase to approximately eight hours after birth, after which there is a reduction.

Although the mitochondria at birth are variable in size—a feature that was not observed in the fetuses—the majority are much larger than those of the 32.5-mm. fetus. The chondriosomes continue to be large and variable in size through the eight-hour stage, then tend to become slightly smaller and more uniform when the filaments are reformed and the vacuoles disappear (figs. 16 and 17).

Two apparently pathological livers were examined, from a four- and a twelve-hour young. The former was obtained alive after both hind legs had been eaten by its mother; the latter appeared normal at the time of killing. Cells of these livers, alike in all respects, are very small, approximately the same size as the 8-mm. embryo, and contain no vacuoles. The ground cytoplasm is dense. All mitochondria are in the form of large, true spheres, no rods, filaments, or hollow spheres being found in any cell of the lobule. They were, no doubt, in a state of heightened activity, and perhaps had consumed the glycogen as well as the non-siderophilic contents of the hollow spheres. No significance can well be attached to these cases, as only two of the eighteen individuals studied in this period were of this type, and the remainder were in stages of the cycle described.

*Two- to eight-day young*

Although the hepatic cells throughout this period may vary intralobularly, the average are approximately the same size as the normal non-vacuolated cells described in the above group. The ground cytoplasm differs from them in that it tends to become darker in cells around the portal unit and that evenly distributed fat vacuoles are found in all livers examined.

At this age the mitochondrial forms presented are exceedingly variable, some cells containing a predominance of polarized, almost smooth filaments (fig. 21); others, spheres and dumb-bells which are located perinuclearly (fig. 20). This variability in configuration of mitochondria is apparently not a cyclic one, since the two most extreme cases found were the same age, namely, five days ten hours. Although the mitochondria of this period are slightly smaller than those of the eighteen-hour young (fig. 17), and the distribution, number, and types of chondriosomes tend to be equal throughout the hepatic lobule, in a few livers cells containing a predominance of spherical mitochondria about one and one-half times as large as those observed in other cells are found scattered through the lobule. These cells resemble the others in regard to size and ground cytoplasm.

It should be noted that the mitochondria of the hepatic cells from two to eight days are extremely variable, as only few of the same age exhibited a similar type of predominating form, and that no true lobular variation, as is present in the adult, is found any livers examined.

Hollow spheres are found in all hepatic cells of these ages except one of five days ten hours and one eight-day young. Up to two days of age these elements, though variable in size, always possess a complete siderophilic cortex, even though they be relatively very large (fig. 1, A to D). However, at the above age the cortex of some of the largest becomes broken, so that instead of being a complete hollow sphere, it is a hollow sphere whose wall is composed of a number of discrete curved segments (fig. 1, G). This process is



not a matter of sudden fragmentation, but of gradual furrowing from the outer surface (fig. 1, E and F). After the fragmentation is completed, the separate segments of the cortex migrate away from the earlier center of the spheres and become individual chondriosomes in the form of curved rods or short beaded filaments, comparable in size to the other elements of the cell, leaving the vacuolar portion of the earlier sphere as an independent structure, which is now seen to be one of the spherical vacuoles, formed by the solution of fat globules, which characterize hepatic cells prepared according to Regaud's procedure. The cortex of these spheres is thinnest just before their fragmentation is initiated. A diagrammatic account of this process is given in figure 1. Stages in

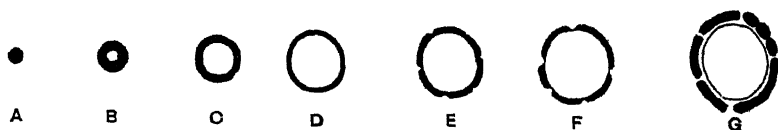


Fig. 1 Diagrammatic presentation of the formation of fat globules within spherical mitochondria. A. Normal spherical chondriosome. B to D. Early stages of the elaboration of fat within the chondriosome. E and F. Fragmentation of mitochondrial covering of fat globule. G. Separation of discrete mitochondrial elements from fat globule.

this manner of formation of fat vacuoles were observed in most of the livers studied after two days of age (figs. 18 to 26).

### *Twelve-day young*

Three definite steps in assuming adult configuration of the hepatic lobule are apparent in the twelve-day liver. One is the growth of all cells of the lobule, so that at this age they are approximately twice as large as those found in the eight-day young, but still slightly smaller than those found in the adult. The ground cytoplasm of the cells bordering the central vein is lighter than it is throughout the remainder of the lobule. There is an increase in the number of mitochondria found in the twelve-day hepatic cell to the extent that, per unit area, the number is equal to that found in the eight-day liver cell, but somewhat less than that found in the adult

liver cell. Spheres, rods, and very short beaded filaments are found in all cells of the lobule, but the cells bordering the central vein have fewer of these types than other cells of the lobule (figs. 23 and 24). This lobular variation is found in all livers examined after twelve days of age.

### *Adult*

Although the hepatic cells of the adult liver differ from those of the twelve-day young in that they are slightly larger in size, they resemble them in the distribution of fat and in the lobular variability of ground cytoplasm; that is, the ground cytoplasm is much lighter in cells immediately surrounding the central vein than it is in the remainder of the lobule. In the hepatic cells of the adult livers examined, the mitochondrial forms presented fell into two more or less distinct groups. While the mitochondria of one group are in the form of long, even, tangled filaments, short beaded filaments, free spheres, and a few short rods (fig. 25), the other have no long, even filaments, but only long, beaded filaments, free spheres, and rods (fig. 26). Relatively few adult livers examined belong to the former group; the majority are either in the latter or in intermediary stages between the two. All cells of one liver have the same types of mitochondria; that is, the various forms of chondriosomes are found throughout the lobule. The adult mitochondrial picture of the former group is first found at fourteen days ten hours of age. Perhaps it is significant that this is about two days before the young start eating the mash fed to the adults, so it is not possible to associate the assumption of adult characteristics with a change of diet. Although this seems to indicate that the first group described is associated with younger individuals, it proved not to be the case, as livers from somewhat senescent males exhibited such extreme mitochondrial variations that both groups were represented and those of nineteen days belong to the second group. Also, there is no correlation as to group and pregnancy. Hollow spheres in various stages of fat formation may be found

in abundance or may be entirely lacking, even though all individuals possess fat in their hepatic cells.

Although the mitochondria of the adult liver cell are approximately equal in size to those of the twelve-day young, there is an increase in their number in the adult, probably brought about by a division of those already present. No lobular variation in mitochondrial size and type can be noticed. There is, however, a distinct lobular difference in relation to numbers. Cells immediately surrounding the central vein have about one-half as many mitochondria as are contained in other cells of the lobule. This scarcity usually involves only one cell layer around the hepatic vein; in rare instances it extends through several. This lobular variability is not being illustrated in figures, because the relative numbers are the same as those illustrated in figures 23 and 24 of the twelve-day young.

It should be noted that, in destaining in excess, scattered cells within the lobule tend to retain the stain with greater tenacity than others, otherwise appearing normal.

#### DISCUSSION

##### *Developmental variations before birth*

Contrary to the observation of Chaves ('20), Arima ('27), and Noël and Pigeaud ('30) on the ontogeny of mitochondria of the hepatic cell of the hedgehog, mouse and man, and man, this study shows that the mitochondria of the developing hepatic cell of the rat undergo changes which are coherent, regular, and definite. The reason that these workers did not find an organized program of mitochondrial change may be due as much to their scanty, scattered material as to the variability which may have been introduced by killing their specimens at various stages of digestive activity or to pathological states of livers. Sake ('27) has shown that a five-day fast in guinea-pigs results in death of the fetuses. This would suggest the likelihood of variable periods of fasting modifying fetal metabolism to the point of altering mitochondrial configuration.

If the data regarding glycogen content of fetal livers of rabbits can be applied to rats, the fetal livers assume individual glycogenic function at seventeen days (Marshall, '22). The glycogen content of the fetal livers would increase at this time and would continue to do so to birth. The above account relative to glycogen formation in fetal livers of the rat entirely substantiates this calculation. The mitochondria during this time are mostly beaded filaments, and, at the time of extremely high glycogen content, twenty days, smooth filaments predominate. According to the work of Noël ('23), Kater ('31), and others, hollow or solid spheres are indicators of heightened function in hepatic tissue. Thus, I would be led to suggest that mitochondrial morphology is not associated with glycogen formation during the first twenty days of intra-uterine life.

If mitochondria of the fetal liver function in bile formation, it would be apparent by enspherulation (according to Noël), thus breaking up the program of filament formation from free spheres which starts at the formation of the hepatic diverticulum and culminates in the twenty-day fetus. Since this course is followed without interruption, and since in the twenty-day fetus, a time when bile formation is probably going on at a rate greater than in previous fetal life, the mitochondria are filamentous, it seems that before birth, at least, chondriosomal morphology is not associated with bile secretion.

Since the changes in mitochondrial configuration in fetal livers cannot clearly be associated with glycogen or with bile formation, it seems highly probable, in view of their coherence in transformation and regularity as to age, that these same transformations are nothing more than one of the aspects of hepatic histogenesis. At the same time we should keep in mind that, even though the regular series of morphological changes may be only a part of histogenesis, it is still possible that the mitochondria are operative during fetal life in somewhat the same manner as in the adult. Whatever this activity may be, it would seem likely that no individual

variability would be found in fetuses of the same age because of the constancy with which nutritive material is supplied to the fetus—a constancy in a much higher degree than characterizes adults. If mitochondria bear any relationship to specific phenomena in adults, we would anticipate that, if those same phenomena occur in fetuses, they would bear the same relationship to them before birth. Since the mitochondria of the hepatic cell are known to vary morphologically with certain aspects of metabolism in adults, the above conclusion relative to histogenesis becomes questionable, unless we assume that hepatic activity in fetuses of all ages is at so low an ebb that it does not influence the mitochondrial configuration characteristic of any histogenetic phase, or that the relationship of mitochondrial form to functional activity is of the nature of a parallel and not a causative agent.

*Developmental and individual variations after birth*

It is evident from the descriptive portion above that there is relatively great individual variation in young of the same age, even though they are litter mates, from parturition to thirty-three hours after birth. Although this variability tends to obstruct any ontogenetic relationship, yet the mitochondrial form and size, as well as cytoplasmic structure of this stage, are distinctly different from those of the fetal or older hepatic cells. One might suggest that the cause for this variation is not a developmental one, but is due to the differences in the length of time between the severance of the placental food supply and killing, a time varying from a few minutes to twenty-four hours. Since these mitochondrial and cytoplasmic changes start in the 32.5-mm. fetus, when the cord is still intact, and are found in one individual twenty-four hours after birth, we can conclude that this thirty-three-hour period is a distinct ontogenetic phase. The physiological alteration associated with this stage of the individual's life that would seem most likely to serve as the underlying cause for this cycle is the disturbance of carbohydrate

metabolism, since this is the period in which glycogen is transferred to the liver from the remaining tissues of the body. Thus mitochondrial morphology is rather definitely associated with glycogenesis. On the other hand, individual variability is probably due to differences in metabolism resulting from such varying factors as temperature, activity, bleeding, and also the individual differences in the available stored food, as Sake ('27) has shown that litter mates may differ greatly in the amount of glycogen located in the liver during the late fetal period.

The hepatic cells of individuals ranging from two to eight days can easily be differentiated from those of any other age on the basis of mitochondrial morphology. Thus, we can regard this period as a distinct development stage of the rat. However, the material observed from two to eight days does not show any sort of morphological mitochondrial cycle, and, because of the wide variation of mitochondrial types presented by individuals of the same age, it seems that the variations within this period are in relation to physiological activity. At the same time the material suggests that this individual variability is caused by something other than digestion, as food was withheld from all individuals an equal length of time. Future investigators studying the function of mitochondria would do well to utilize material of this or similar ontogenetic phases, because if one were to succeed in isolating the factors causing this great variability, one would be obtaining significant evidence with reference to the nature of mitochondrial activity.

The fact that an intralobular differentiation of mitochondria, with respect to number, is found in the twelve-day young similar to that found in the adult indicates, I believe, that at this early age chondriosomes of the liver are stabilized with respect to functional activity, and alterations in number and types are modifications made to continue this activity at variable levels.

The individual variations in the adult livers are extremely slight, as can be noted from the above description, the vari-

ability being limited to two types and their intermediate stages, one type being characterized by smooth filaments, the other by beaded filaments. It would seem quite evident that this aspect of mitochondrial variability in the hepatic cell is entirely unrelated to digestive activity, as the conditions—namely, regularity of feeding, food material, and period of starvation previous to killing—were the same in all cases. At the same time, it would be difficult to conceive the underlying cause for this variability, and, until this is done, it would be impossible for one to obtain perfectly controlled conditions for studies on hepatic mitochondria.

### *Morphology of mitochondria*

Contrary to the theory of the continuity of definite genetic types of chondriosomes, the material examined in this study shows that spheres become associated, forming beaded filaments, which in turn become long, smooth ones. Regular filaments may become beaded, and the beads become separated, forming spheres, and hollow spheres fragment to form rods or beaded filaments. In this way, any form of mitochondria may change into any other form. This beading of filaments, according to some writers, is attributed to faulty fixation. Due to the fact that in the twenty-day fetus and in the adult both smooth and beaded filaments are found in the same hepatic cell—an improbability if poor fixation were the cause—and since the Lewises ('25) have observed in living cells the fragmentation of beaded filaments to form spheres, the only possible conclusion is that beaded filaments are a natural condition of mitochondria and constitute a transition form between spheres and smooth filaments. Taniguchi ('28) has described beaded filaments in the hepatic cell of man, and regards them as normal.

Students of mitochondria have been prone to consider that the same organ in the same species or nearly related species will possess mitochondria of the same configuration. Thus Cowdry ('25) says:

The cells of the liver, pancreas, lungs, and other organs possess mitochondria which are alike in nearly related animals. This constancy in shape where the function is similar indicates that the morphology of mitochondria is a fundamental property engrained in the organization of the cell, and that it is not always a passing, trivial affair which varies from moment to moment.

Although this concept may have an element of truth, it is evident from the present study that it is not true in reference to ontogeny, since the characteristic mitochondrial configuration of the liver at one stage of development is very unlike that of another stage. In the second place, when we consider the adult mitochondrial picture of the white rat and compare it to the adult mitochondrial picture of nearly related animals, such as the mouse, and a somewhat distantly related one, such as the cat, we find that this assertion still does not hold true. In the rat, as is described above, there is no intralobular variation except in reference to numbers, and the characteristic mitochondrial form is a combination of beaded filaments, short rods, and spheres. In the white mouse, with the same conditions in regard to digestive activity, Noël ('23) finds that the mitochondria of the hepatic lobule vary from long, smooth, undulating filaments at the central vein to short rods at the portal vein, with a gradual transition in the intervening area. Under the same conditions Kater ('31) finds the mitochondria of the hepatic lobule of the cat to vary from long, smooth filaments bordering the portal unit to short rods at the central vein. According to both of the accounts, the mitochondria of any one cell are all of approximately the same type. Thus, the mitochondria of the hepatic lobule of the mouse and the cat are essentially the same, except that the distribution of the various types is reversed. In both of these animals the picture is entirely different from that described in the rat.

#### *Nature of mitochondrial activity*

The nature of mitochondrial activity is naturally the objective for all studies on mitochondria. This objective



has proved to be an elusive one, perhaps because of the necessity of relying entirely upon objective cytology, which has given us no more than accounts of mitochondrial variability associated with more or less known physiological states. As a result of this rather vague approach to the problem, quite a variety of suggestions as to the nature of mitochondrial functions have been made. These range from the idea that mitochondria may be directly transformed into protoplasmic bodies such as plastids, or deutoplasmic bodies such as yolk and fat, to the idea that they are catalytic agents influencing chemical reactions within the cell. Still another concept leaves their functional significance as a total enigma and interprets their morphological changes as being a passive result of physical conditions in the cell, thus regarding them as markers of general metabolic activity.

The observations of the present work, it seems to me, significantly reflect on this problem, particularly in reference to the formation of fat. The fact that fat globules are formed in the substance of spherical chondriosomes, to be later liberated in the ground cytoplasm by the fragmentation of their mitochondrial covering, must be interpreted in one of two ways, either the mitochondria serve as a storage place for fats or act as catalysts stimulating a synthesis of fats from the fatty constituents. The non-diffusibility of fats makes the former seem very unlikely. Thus, I am led to conclude that the above description constitutes very strong evidence for the catalytic nature of mitochondrial activity, at least in reference to fat formation. This is rather clearly contradictory to the idea that mitochondria become converted directly into fat (Schreiner, '15).

It is quite evident, however, that this is not a complete solution of the problem even with reference to the hepatic cells of the white rat, since very striking cyclic changes of the morphology of the mitochondria parallel the formation of fat within some of the spheres. These other transformations, such as the formation of beaded filaments from spheres and smooth filaments from beaded filaments, or changes in

the opposite direction, most assuredly cannot be regarded as being related to the formation of fat. And since there is no evidence of the accumulation of storage products other than fats in these types of mitochondria, we are left without any concrete evidence as to the cause of their transformations. For instance, the striking mitochondrial changes that occur immediately after birth could well be a result of chondriosomal activity in glycogen synthesis; at the same time it is equally possible that these same changes are caused by the physical state of the cell and are merely parallels of the synthetic activity. In view of the variety of phenomena that are known to influence the form of mitochondria, perhaps we should favor the latter—a suggestion that has already been made in reference to the hepatic cell by Kater ('31). Noël ('23) considered the hollow spheres of the hepatic cell of the mouse to be related to bile formation. From his description they are similar to those described in the present account as being active in fat formation. The same worker also thought the mitochondria of the liver to be associated with fat formation, but his evidence consisted of a hypertrophy and enspherulation of chondriosomes in the vicinity of developing fat globules—the same type of evidence that I have obtained with reference to glycogen formation.

#### SUMMARY

All animals were starved twenty-four hours before killing, except the young from birth to twenty-four hours, which were taken from the mothers at delivery.

The mitochondria of the cells of the hepatic diverticulum of the 3-mm. rat embryo are in the form of minute spheres. In older embryonic hepatic cells these spheres enlarge and become associated to form beaded filaments, which, in turn, become smooth filaments. This process culminates in the twenty-day fetus, one day before birth, when smooth filaments predominate. Hollow spheres appear in the hepatic cell of the seventeen-day fetus and are invariably present up to the eight-day young; after that age they occur intermittently.

All fetuses of the same age present the same mitochondrial picture as to number, type, and size.

Starting one-half day before birth, the formerly smooth mitochondrial filaments of the hepatic cell are found to be distinctly beaded, and the beads become dissociated, forming spheres; although the size of all types of mitochondria is variable, there is an enlargement of beaded filaments and spheres. The high point of this cycle is attained at approximately eight hours of age, when large spheres are in predominance. After that age spheres are associated to form beaded filaments again; and there is also a regression of size. After birth, young of the same age, even though they be litter mates, may not present the same mitochondrial picture, yet the hepatic cells of all individual livers studied between parturition and thirty-three hours of age show mitochondria of some part of this particular ontogenetic cycle.

The chondriosomes of the hepatic cells of the two- to eight-day young are extremely variable both as to types and size.

At twelve days of age there is a marked increase in the number of mitochondria in all cells of the hepatic lobule except those immediately around the central vein. Very short beaded filaments, rods, and spheres are found in equal proportion in all cells of the lobule. The mitochondrial configuration of the adult hepatic lobule is first found in the fourteen-day ten-hour young. There are two general types, one in which all of the cells contain long beaded filaments, rods, and spheres and one in which all cells contain long, even filaments, rods, and spheres. Although there is no localization with reference to number of mitochondrial types, there is a lobular localization with reference to number of mitochondria. Cells around the central vein have fewer of all mitochondrial types than any other cells of the lobule.

The fact that the mitochondria of the hepatic cells of fetuses of the same age are the same as to type and size, and since the mitochondrial transformation of developing fetal hepatic cells are uniform and regular, it is strongly indicated that before birth the mitochondrial changes in size and shape are

histogenetic changes. Though mitochondria may be functional before birth, the histogenetic changes seem to be in control.

It is suggested that the ontogenetic phase of mitochondria found immediately after birth is in relation to the disturbance of the metabolic equilibrium existing before birth and that individual variation in this same period is the result of individual variation in such conditions as amount of stored food, bleeding, temperature, etc. The principal aspect of such a disturbance that has been observed is the accumulation of quantities of glycogen in the hepatic cell at the time of birth.

The mitochondrial differences in hepatic cells of two- to eight-day young represent histogenetic and functional variations other than those associated with digestion.

The adult livers examined indicate that some factor other than age, type of food, period of digestion, and length of starvation plays a part in mitochondrial forms found in the hepatic cell.

The conversion of solid spherical chondriosomes into hollow spheres results from the accumulation of fat within them. These fat globules enlarge and their mitochondrial covering fragments, forming rod-like mitochondria and liberating the fat globules in the ground cytoplasm. It is pointed out that this constitutes strong evidence for the catalytic nature of mitochondrial activity with reference to fat formation.

I wish to express my appreciation to Dr. J. McA. Kater for his many helpful suggestions and criticisms.

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## PLATE

### EXPLANATION OF FIGURES

All figures were drawn from  $4\mu$  sections of liver, prepared according to the method of Regaud, under Leitz fluorite oil-immersion objective (1/16) and compensating ocular (15 $\times$ ), with the aid of Abbé-model camera lucida. All material was obtained from animals which had not been fed for twenty-four hours, except those represented in figures 10 to 14, 16, and 17, which were taken from the mothers at delivery.  $\times 1860$ .

## PLATE 1

### EXPLANATION OF FIGURES

2 to 8 Note the enlargement and association of spheres to form beaded filaments which become smooth filaments in figure 8. Hollow spheres and irregular vacuoles (glycogen) appear in figure 6.

2 Cells of the hepatic diverticulum of a 3-mm. rat embryo.

3 Hepatic cells of an 8-mm. rat embryo.

4 Hepatic cell of an 11.75-mm. rat embryo.

5 Hepatic cells of a 13.5-mm. rat fetus.

6 Hepatic cell of a 16.5-mm. rat fetus.

7 Hepatic cell of a 22-mm. rat fetus.

8 Hepatic cell of a 28-mm. rat fetus.

9 to 17 Figure 9 is distinguished from figure 8 by an enlargement of mitochondrial elements, a conversion of smooth to beaded filaments, a separation of the beads to form spheres, and an enlargement of the irregular vacuoles (glycogen). These changes continue in figures 10, 11, 12, 14, and 15. As the vacuoles become smaller the mitochondria appear in beaded filaments (figs. 13, 16, and 17). Hollow spheres, variable in size, are present in all cells.

9 Hepatic cell of a 32.5-mm. rat fetus.

10 Hepatic cell of rat at birth.

11 Hepatic cell of one-hour young.

12 Hepatic cell of eight-hour young.

13 Hepatic cell of eight-hour young.

14 Hepatic cell of twelve-hour young.

15 Hepatic cell of twenty-four-hour young.

16 Hepatic cell of eighteen-hour young.

17 Hepatic cell of eighteen-hour young.

18 to 22 The fragmentation of the mitochondrial covering of the hollow spheres (fat globules) is apparent in figures 18 to 20. Note the variability in mitochondrial forms throughout the period.

18 Hepatic cell of a two-day young.

19 Hepatic cell of three-day eight-hour young.

20 Hepatic cell of five-day ten-hour young.

21 Hepatic cell of five-day ten-hour young.

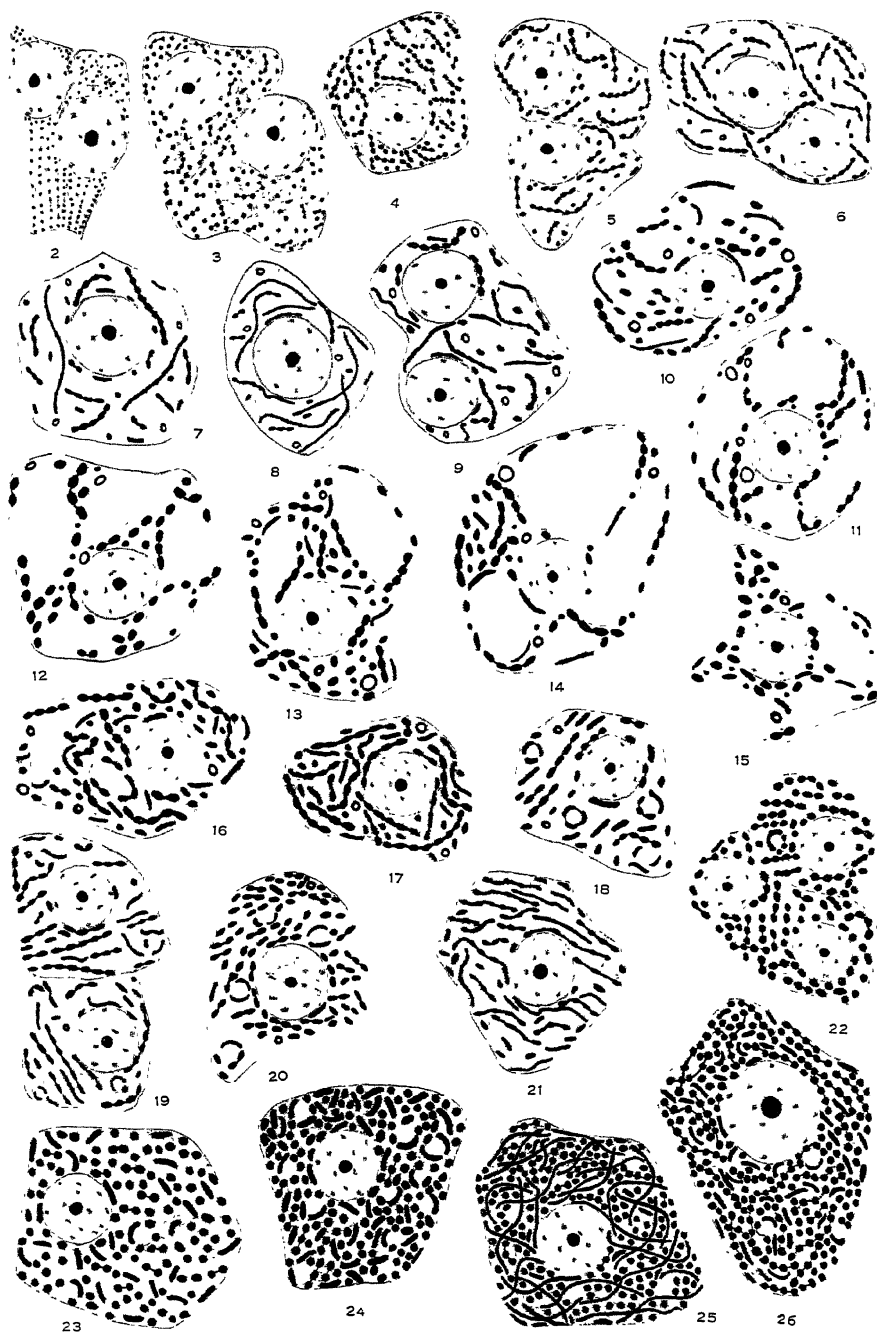
22 Hepatic cell of eight-day six-hour young.

23 Hepatic cell bordering the central vein of a twelve-day four-hour rat.

24 Hepatic cell bordering the portal unit of the same lobule.

25 Hepatic cell bordering the portal unit of an adult rat.

26 Hepatic cell bordering the portal unit of another adult rat.







# THE DEVELOPMENT AND THE REGENERATION OF THE COLOR PATTERN IN BRACHYDANIO RERIO

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TWO TEXT FIGURES AND TWO PLATES (FOURTEEN FIGURES)

## AUTHORS' ABSTRACT

The teleost fish *Brachydanio rerio* is strikingly marked with longitudinal black stripes, which extend into the caudal fin and across the anal fin. Removal of the anal fin is followed by complete regeneration of the fin and of its normal color pattern. Microphotographic studies show that melanophores are at first uniformly distributed in the regenerating tissue and that later the melanophores disintegrate in the zone of the future light stripe and increase in the region of the future dark stripe. Observations on the normal development of the fin where the history of the individual melanophores has been followed show the same mode of formation of the stripes.

In this paper a description is given of the formation of the color pattern in a tropical fish. The study has been made in connection with a program dealing with the development of mendelian characters and color patterns of fish now being undertaken in the Laboratory of Biology at Wesleyan University.<sup>1</sup>

## MATERIALS AND METHODS

The fish used in these experiments, *Brachydanio rerio*, Hamilton and Buchanan, popularly called the zebra fish and well known to fish fanciers, is a native of Celon. It is placed by Jordan ('23) in the family Cyprinidae, of the order Eventognathi. It averages  $1\frac{1}{2}$  inches in length and bears striking black and yellow longitudinal stripes (fig. 1). These stripes are formed by pigment cells which lie in the dermis

<sup>1</sup> This program is made possible by grants from the Denison Foundation for Biological Research at Wesleyan University. Preliminary observations were made by one of the authors (H. B. G.) at the Kaiser Wilhelm Institut and he wishes to make grateful acknowledgment of the facilities provided at that time. We are indebted to Mr. Norris Jones for figure 1.

beneath the scales. Scales when removed are without pigment cells. The fact that the stripes run horizontally in the fins suggested that the fish might be useful in studying regeneration of the color pattern, because by amputating the anal fin it would be possible to observe regeneration in a direction perpendicular to the striping and removal of the caudal fin would show regeneration along the axis of striping.

The removal of the fin is a simple operation. While anaesthetized by chloretone the fish is laid in a wax depression and the fin is cut off with fine scissors. This species is notably gregarious, and therefore the individuals were not isolated, but several were kept together in large culture dishes. Under these conditions the fish are more vigorous than when isolated and the regeneration proceeded more rapidly. The fish were kept in a room at an approximate average temperature of 21°C. Photographs were taken with a Leitz "Makam" camera and in some cases have been retouched, as it proved almost impossible to obtain a sufficiently flat field to show all chromatophores at the same focal level. Those slightly out of focus have been accentuated.

The young fish used in observations on normal development were reared in our laboratory. They are easy to raise and are quite hardy. At different intervals the fish were anaesthetized with TS 222<sup>2</sup> and records were made, by means of a microprojection apparatus, of the growth and melanophore formation of the anal fin. A drop of adrenalin solution (1:10,000) was applied to the fin, to induce contraction of the chromatophores and to make their outlines more clear-cut. Observations were in each case limited to the region of a single ray. This restriction of area was necessary in order to reduce the duration of the period of anaesthesia. It was necessary to segregate these fish for purposes of individual identification, but as a result they showed a marked retardation in development when compared with fish of the same age

<sup>2</sup> This anaesthetic was kindly provided by the Pharmakologisches Laboratorium, Chemische Fabrik vormals Sandoz, of Basel, Switzerland. It is known only by the trade name given above.

kept together in a large aquarium. At the age of five months the segregated fish averaged 20 mm. in length, while the non-segregated controls were 30 mm. in length. No further analysis has been made of the factors involved in this differential growth, but there are apparently some beneficial effects of the animal aggregation, such as are reviewed by Allee ('31).

#### REGENERATION OF COLOR PATTERN IN FIN OF THE ADULT FISH

The regeneration of both the caudal and the anal fin restored the original pattern in practically all cases. The formation of the pattern in the caudal fin involved merely the prolongation of the longitudinal body stripes and seemed of less interest than the corresponding process in the anal fin, to

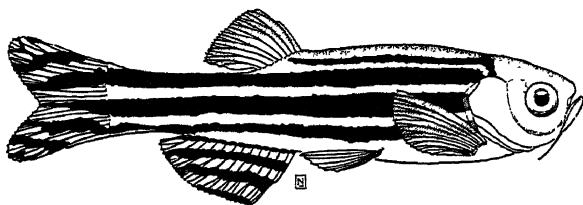


Fig. 1 *Brachydanio rerio*, adult male. Showing a regenerated caudal fin in which the stripes are not as regular as in a normal fish.  $\times 2$ .

which our attention has therefore been chiefly directed. It will be realized by inspection of figure 1 that the regeneration of the anal fin involves the formation of isolated stripes and not merely the prolongation of stripes already present.

An attempt has been made to follow the history of the melanophores during the process of stripe formation in the regenerating anal fin. A week after an operation a series of photomicrographs was begun and thereafter photographs were taken, usually at two-day intervals, until the regeneration of the fin was completed. Figures 3 to 8 are selected from one of these series.

In the earliest stage the stump of the old fin is recognizable because of its pigmentation, and beyond this is the clear, transparent, and as yet unpigmented regenerating tissue (fig.

3). The photographs (figs. 4, 5, 6) show that the melanophores increase continuously as the fin grows. After about three weeks, the fin is approximately one-half grown and is almost uniformly dotted with melanophores (fig. 6). Shortly after this stage is attained, a marked thinning out of melanophores begins in a horizontal band near the base of the fin (fig. 7). A degeneration of melanophores continues until a zone has been practically cleared (fig. 8). During the process of melanophore destruction the xanthophores begin to appear in the clear zone and initiate the process of formation of the yellow stripe. Simultaneously with these events, the growth of the fin has continued distally. The rays have lengthened and more melanophores have appeared in the growing tissue. At the same time a greater concentration of black cells appears on either side of the clear zone. These are the developing black stripes. During this period the melanophores assume their adult condition. The physiological and morphological difference between the young and the mature type of melanophores is obvious. In young cells the melanin concentrates readily under the adrenalin treatment, whereas in mature cells the melanin is but slightly affected, remaining widely distributed in the amoeboid processes of the cell.

If a second yellow stripe forms, the process described above is repeated.

#### DEVELOPMENT OF THE COLOR PATTERN IN THE ANAL FIN OF YOUNG FISH

Melanophores appear in the anal fin prior to the appearance of fin rays and to the establishment of the circulation in the fin. At this stage they are few in number. As growth continues and the rudiments of the rays appear the melanophores tend to concentrate at their distal ends (fig. 2). Eventually, however, when the rays lengthen and begin to segment, the pigment cells also line up along either side of the rays (fig. 2). At this stage the melanophores are evenly distributed throughout the fin and this arrangement is strictly comparable to the similar, previously described, phase in the regenerating fin. Soon the melanophores in the proximal

region of the fin begin to disappear (fig. 13), and simultaneously the yellow cells or xanthophores are first recognized, appearing in this same region. The destruction of the melanophores appears to be somewhat fluctuating, as if there were antagonistic influences, and a period of a few days elapses before an equilibrium is reached. Individual cells apparently disappear, then reappear, but finally disintegrate. This phenomenon of fluctuation is more obvious than in the regenerating fin. At no time have observations indicated that migration of melanophores plays any part in stripe formation. The melanophores beyond the yellow stripe increase in number (fig. 14), forming the black stripe, and distal to this more black cells appear evenly distributed,

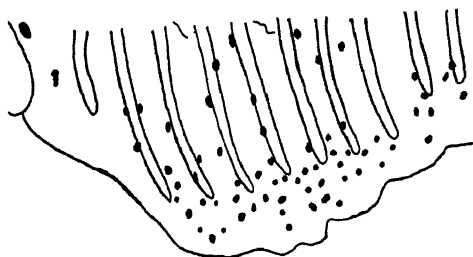


Fig. 2 Anal fin of a young fish with melanophores along the rays and concentration of these cells at the distal ends of the rays.  $\times 55$ .

presaging a second critical period of degeneration which will make room for the second yellow stripe. The duration of these various events is indicated by dates on the figures, which have been chosen from a long series of observations made at two-day intervals.

#### DISCUSSION AND SUMMARY

This mode of formation of the color pattern in part suggests that known to exist in the goldfish *Carassius auratus* (Koketsu, '15; Berndt, '25; Fukui, '27 and '30; Goodrich and Hansen, '31). This consists in the first development of a uniform self-coloring, followed by a localized destruction of chromatophores, leaving a color pattern. In the goldfish

the pattern of disintegration differs with each individual fish. In *Brachydanio rerio* the pattern is regular and practically identical in all fish. There appears, however, in this species a second process apparently not present in the goldfish. This is the formation, after the period of disintegration, of additional melanophores in the region of the dark stripes. In this respect it resembles more closely the condition found in *Oryzias latipes* (Goodrich, '27), where no destruction occurs and the darker cells appear merely to congregate in certain regions to form a diffuse pattern.

Two processes are then concerned in the formation of the stripes in the fin of *Brachydanio rerio*. These are the partial destruction of an earlier uniform self-coloring and the later accumulation of additional melanophores in the dark stripes. Xanthophores appear in the light zones as the melanophores disintegrate. These phases can be traced both in the normal development and in the regenerating fin.

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## PLATES



## PLATE 1

### EXPLANATION OF FIGURES

The figures of this plate are photomicrographs of the regenerating anal fin of fish no. 1. The anal fin was removed on October 9, 1930. The cut was made through the second black stripe from the body.

3 Anal fin showing transparent regenerated tissue beyond the stump of the old fin. October 22, 1930.  $\times 40$ .

4 A selected area including rays 2, 3, and 4 showing melanophores. October 22, 1930.  $\times 90$ .

5 The same area. October 25, 1930.  $\times 90$ .

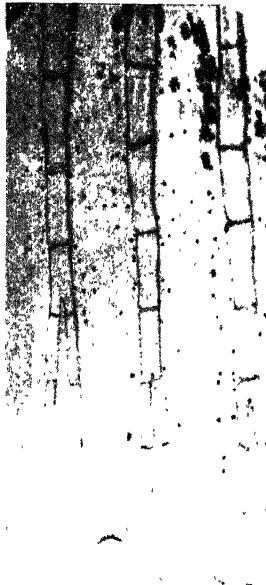
6 Concentration of melanophores at the distal ends of the rays. October 31, 1930.  $\times 90$ .

7 The breakdown of melanophores where the yellow stripe is going to form. November 15, 1930.  $\times 90$ .

8 Region of the yellow stripe completely cleared and the black stripe present on each side. November 22, 1930.  $\times 90$ .



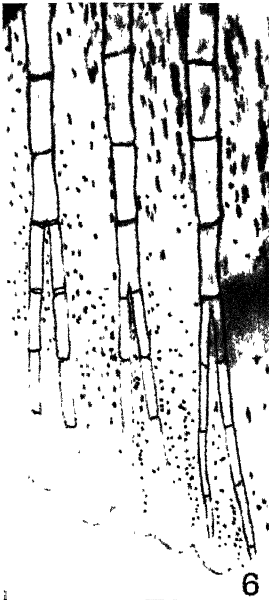
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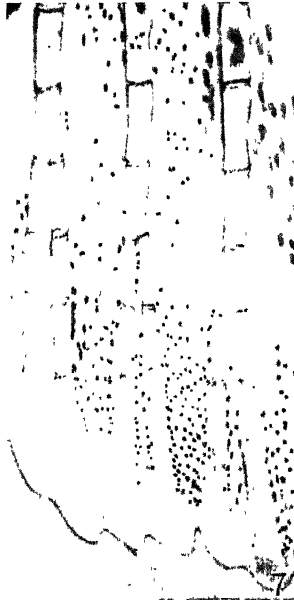
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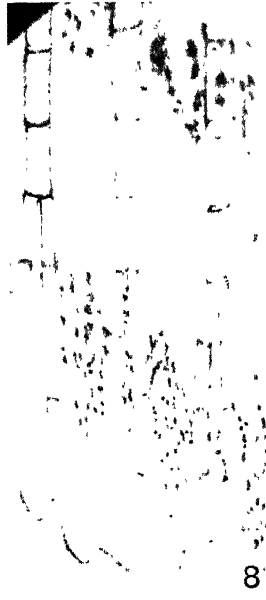
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## PLATE 2

### EXPLANATION OF FIGURES

The following series of figures were drawn with a microprojection apparatus. Drawings are of the region of the fifth ray of the anal fin of young fish U. Magnification of all figures is  $\times 55$ .

9 Uniform melanophore distribution along the ray. January 9, 1931.

10 Breakdown of melanophores in middle region of the ray. January 24, 1931.

11 Further melanophore destruction in middle region. February 6, 1931.  
 $\times 55$ .

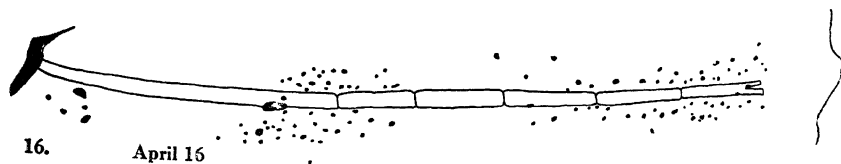
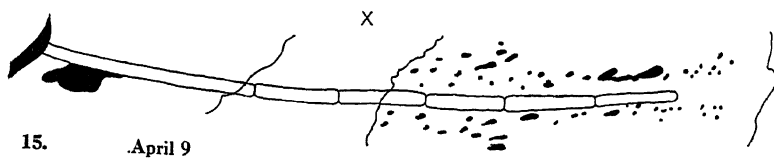
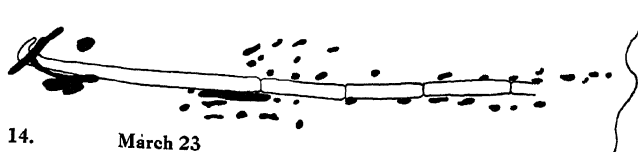
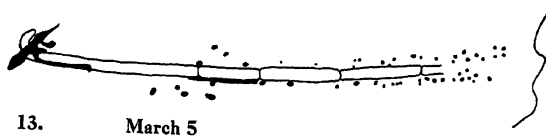
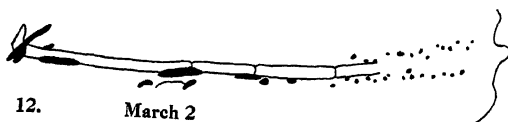
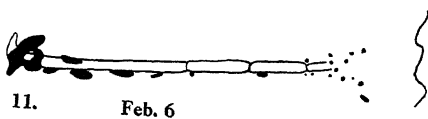
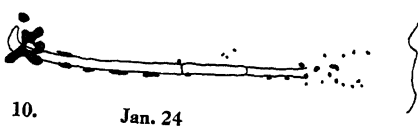
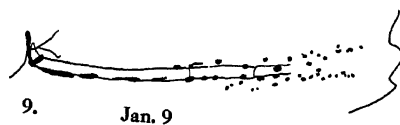
12 Melanophore destruction at the proximal end of the ray and concentration at the distal end. March 2, 1931.

13 Increase in the number of melanophores in the region of the second segment to form first black stripe. March 5, 1931.

14 Increase in the length of the ray and number of melanophores to form the first black stripe. March 23, 1931.

15 X marks the first black stripe the melanophores of which were not contracted, so presented a diffuse dark area which is outlined. The uniform distribution of black cells in the distal region is characteristic of this stage. April 9, 1931.

16 Melanophore destruction distal to the first established black stripe clearing a region for the second yellow stripe. The basis for two yellow and two black stripes is established. April 16, 1931.





# CHROMOSOMES OF THREE SPECIES OF MANTIDAE

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TWO PLATES (TWENTY-THREE FIGURES)

## AUTHOR'S ABSTRACT

There are twenty-seven spermatogonial chromosomes in *Tenodera sinensis*, three of which are larger than the others and lag behind in anaphase. These three chromosomes have been traced through the prophases of the first spermatocyte to the metaphase, where they appear as a hexad. In the first spermatocyte division two of the constituent diads of the hexad pass to one pole, the remaining one to the other, thus giving rise to two types of second spermatocytes, one with thirteen and the other with fourteen chromosomes. There are twenty-eight chromosomes in somatic metaphases of the female; four of these chromosomes are larger than the others. The sex chromosomes of the male are represented by the formula  $X_a + Y + X_b$ , that of the female by  $2X_a + 2X_b$ .

Similar conditions are reported for *Mantis religiosa* and *Stagmomantis carolina*.

## INTRODUCTION

So far as the writer is aware, there have been only two contributions to the cytology of the Mantidae: Giardina ('97) reported the chromosomes of *Mantis religiosa* and Oguma ('21), those of *Tenodera superstitiosa* and of *Paratenodera aridifolia*. This paper will consider only the chromosomes of three species of Mantidae; the cytoplasmic components will be dealt with in a later paper.

## MATERIALS AND METHODS

This investigation has been made upon *Tenodera sinensis*, *Mantis religiosa*, and *Stagmomantis carolina*, all of the subfamily Mantinae. *Tenodera sinensis*, the Chinese mantis, was obtained from the vicinity of Philadelphia, Pennsylvania, where it has become established since its introduction about 1897 in nursery stock. Testes of this species (sixty-two individuals, collected 1921 to 1928) were fixed entire in Allen's B<sub>15</sub>, Bouin's, or Flemming's solution, cut into sections, and stained in safranin or iron-hematoxylin. Smears were made according to the method described by McClung ('29), fixed

in Flemming, and stained in safranin or iron-hematoxylin. It may be of interest to know that fixation of smears is usually very superior and that the whole history of the chromosomes may be traced in properly made smears. Entire ovaries were fixed in Bouin's or Allen's B<sub>15</sub>, sectioned, and stained.

Ten specimens of *Mantis religiosa*, the European mantis, were obtained in August, 1928, on the campus of Cornell University, Ithaca, New York, and fixed entire in modified Bouin's and in Minouchi's F.w.a. solutions. They were sectioned and stained in iron-hematoxylin.

Three specimens of *Stagmomantis carolina* were obtained from the collection of Prof. C. E. McClung, of the University of Pennsylvania, to whom I am indebted for the use of this material. Professor McClung has in his possession a photograph of a number of cells from one of the earlier preparations of this species. Such is the technical excellence of the preparation that the same location he photographed over thirty years ago may still be identified and studied at the present time. Two of the individuals were fixed in Flemming, the other in Bouin's with the addition of chromic acid; sectioned, and stained in iron-hematoxylin.

#### OBSERVATIONS

##### *Tenodera sinensis*

There are twenty-seven atelomitic chromosomes in the spermatogonial metaphase, two of which are conspicuously larger than the others and usually show a terminal diffuse condition (figs. 3, 4). One of these chromosomes has a median fiber attachment; the other, an intermediate one. These two and one other smaller element fail to pass directly to the pole of the cell in anaphase (fig. 5), but lag behind and eventually form a separate vesicle (fig. 6), which finally fuses with the telophase nucleus. These chromosomes which have lagged in division remain dense long after the others have become very diffuse (fig. 7). They stretch out over the surface of the nucleus (figs. 8, 9), and finally in late prophase

two of them become diffuse, one remains dense, swells as the cell increases in size, and becomes the most conspicuous element in the cell (figs. 10, 11). In addition to this large nucleolus-like structure, there is a fairly large deeply staining karyosome which often appears double (figs. 10, 11). This karyosome later fails to take the chromatin stain and becomes a plasmosome. At diakinesis the plasmosome has disappeared and the large nucleolus which had its origin from one of the lagging chromosomes appears with a more diffuse element on either end (figs. 12, 13, 14). This tripartite structure may be traced into the first spermatocyte as the hexad shown at the extreme left of figure 15. This hexad may be interpreted as a sex-determining mechanism of the constitution  $X_a-Y-X_b$ .  $X_a$  corresponds to the larger chromosome with median fiber attachment in the spermatogonia,  $X_b$  to that with an intermediate one, and Y to the other smaller chromosome which also lags in spermatogonial anaphase. It is evident that the large nucleolus present in late prophases has its origin from the element here designated as Y. The twelve other chromosomes present in the first spermatocyte are tetrads each of which is made up of homomorphic elements, corresponding to the twenty-four atelomitic chromosomes found in spermatogonia.

In the first spermatocyte division each of the twelve tetrads divides; the hexad divides also, with the two larger elements  $X_a$  and  $X_b$  passing to one pole and the smaller Y to the other. Infrequently the hexad seems to divide so that one X and Y go into one daughter cell and the other X into the other daughter cell. The usual type of division gives rise to two types of second spermatocytes: those with  $12 + X_a + X_b = 14$  chromosomes (fig. 18) and those with  $12 + Y = 13$  chromosomes (fig. 21). In the second spermatocytes each chromosome divides, so that there are two types of sperm corresponding to the two types of second spermatocytes. The second spermatocytes originating from the unusual type of division of the hexad in the first spermatocyte have not been distinguished.



There are twenty-eight chromosomes in the somatic cells of the female, of which four are conspicuously larger than the others (fig. 1). Two have median fiber attachment, as has  $X_a$  of the spermatogonia; the other two have intermediate fiber attachment, as has  $X_b$ . However, these elements do not show the differential behavior in anaphase which their homologues show in the spermatogonia anaphases (fig. 2). The chromosome constitution of the female, then, is interpreted as  $24 + 2X_a + 2X_b$  and that of the male as  $24 + X_a + X_b + Y$ .

### *Mantis religiosa*

No spermatogonial divisions of this species were available, but diakinesis stages show a large tripartite element similar to that in *Tenodera sinensis*. The first spermatocytes (fig. 16) show thirteen chromosomes, one of which is a hexad. Here the Y has median fiber attachment, while both  $X_a$  and  $X_b$ , which seem unequal in size and smaller than the Y, have intermediate fiber attachment. The chromosomes divide in the first spermatocyte division, as in *Tenodera sinensis*, and give origin to two types of second spermatocytes, one with fourteen chromosomes (fig. 19), the other with thirteen (fig. 22). The second spermatocytes give rise after division to two corresponding types of spermatozoa.

### *Stagmomantis carolina*

No spermatogonial divisions of this species were available, but the prophases of the first spermatocyte show a differential tripartite element, as in *Tenodera sinensis* and *Mantis religiosa*. The first spermatocyte metaphases contain thirteen chromosomes, one of which is a hexad (fig. 17). Here the Y chromosome is a small spherical body, while  $X_a$  and  $X_b$  are large, with approximately median fiber attachment. The chromosomes divide as in the two other species, resulting in second spermatocytes with fourteen (fig. 20) or thirteen chromosomes (fig. 23). The sperm would be of the same two classes.

## DISCUSSION

The chromosomes of *Mantis religiosa* were studied by Giardina ('97), who found fourteen chromosomes in the spermatogonial and in the first spermatocyte metaphase. No observations were reported of any differential chromosomes corresponding with  $X_a$ -Y- $X_b$ . This is not surprising, in view of the fact that many of the pioneer workers, especially those whose material was difficult, overlooked what has since been made obvious by improved methods and different emphasis.

*Tenodera superstitiosa* and *Paratenodera aridifolia* were studied by Oguma ('21), who found conditions essentially similar to those described here for *Tenodera sinensis*, *Mantis religiosa*, and *Stagmomantis carolina*. Oguma considers that the idiochromosomes  $X_a$ -Y- $X_b$  immediately after the last spermatogonial division "coalesce at last into a single body," which "coalescence . . . can, however, be nothing more than a simple mechanical fusion" ('21, p. 8). As described here,  $X_a$  and  $X_b$  become diffuse, while Y undergoes heteropycnosis. This difference of opinion may be due to the fact that Oguma has not studied the early prophases of the first spermatocytes in which no evidence of this fusion is found. Studies of these stages are best pursued upon material fixed by the smear method, where fixation of these stages is superior to that in testes fixed entire.

For a discussion of sex chromosomes in general the reader may be referred to Schrader ('28).

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## EXPLANATION OF PLATES

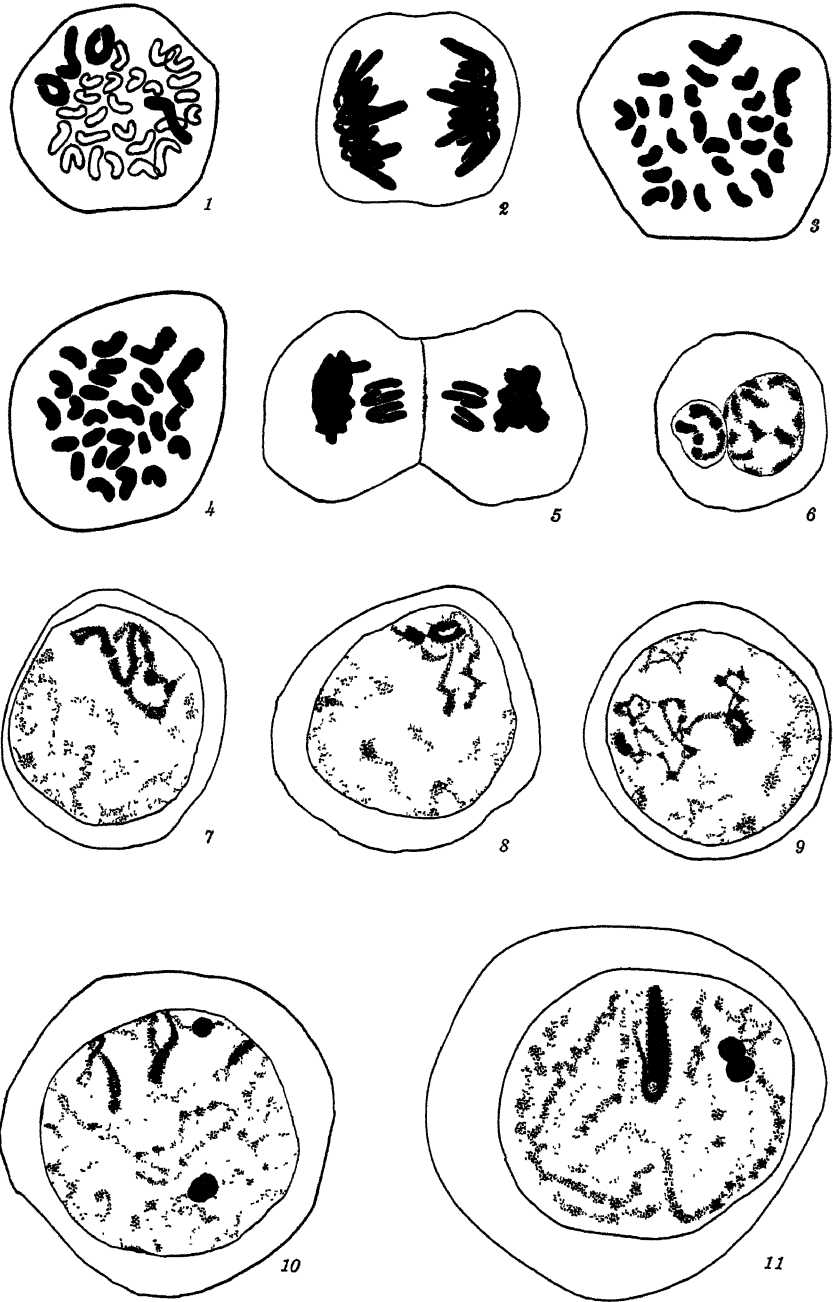
Figures reproduced at 2000 diameters except where otherwise stated.

### PLATE 1

#### EXPLANATION OF FIGURES

##### *Tenodera sinensis*

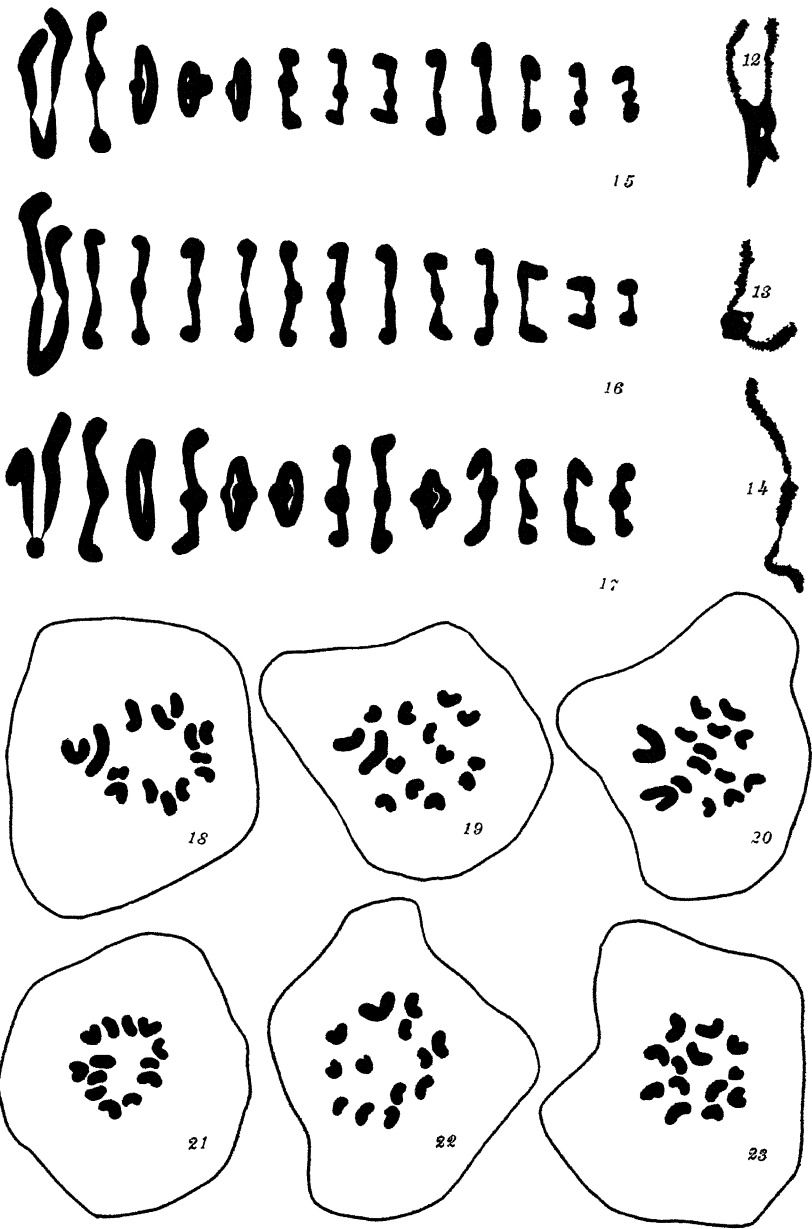
- 1 Metaphase of follicular cell of female.  $\times 3000$ .
- 2 Telophase of follicular cell of female.  $\times 3000$ .
- 3 and 4 Spermatogonial metaphases.
- 5 Early spermatogonial telophase.
- 6 Later spermatogonial telophase.
- 7 to 11 Prophases of first spermatocyte.



## PLATE 2

### EXPLANATION OF FIGURES

- 12 to 14 Sex chromosomes in diakinesis, *Tenodera sinensis*.
- 15 to 17 Chromosomes rearranged to show form.
- 15 First spermatocyte metaphase, *Tenodera sinensis*.
- 16 First spermatocyte metaphase, *Mantis religiosa*.
- 17 First spermatocyte metaphase, *Stagmomantis carolina*.
- 18 to 20 Second spermatocyte metaphases, fourteen chromosomes.
- 18 *T. sinensis*.
- 19 *M. religiosa*.
- 20 *S. carolina*.
- 21 to 23 Second spermatocyte metaphases, thirteen chromosomes.
- 21 *T. sinensis*.
- 22 *M. religiosa*.
- 23 *S. carolina*.





# MODIFIED MITOSIS IN THE CHICK EMBRYO

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EIGHT PLATES (SIXTY FIGURES)

## AUTHOR'S ABSTRACT

A study of cell division in the chick embryo has revealed in striated muscle and in the early stages of all the principal tissues and organs a process, followed by the greater percentage of the dividing nuclei, which resembles amitosis, but is undoubtedly a highly modified form of mitosis. This process is designated 'modified mitosis.' The belief that this is essentially mitotic rests upon the fact that a number of discrete bodies may be distinguished in the one or two chromatin masses of each nucleus. These masses, or 'mulberries,' might easily be mistaken for nucleoli or karyosomes were it not that they exhibit division by elongation and constriction. These structures probably represent masses of minute and closely clumped chromosomes in somatic number dividing in a qualitative and a quantitative fashion. As additional evidence there are present between the dividing mulberries exceedingly fine fibers which possibly represent an attempt at spindle fibers. Neither centrosomes nor asters are present, but since, if present, they would have to be of the intranuclear variety (the nuclear membrane never disintegrating) their absence is immaterial. The nucleus divides not by constriction, but by the formation of a nuclear plate. This process of modified mitosis is probably a device which favors the speed of nuclear proliferation necessary to such rapidly growing tissues.

It often occurs that old theories may be restudied with profit. Some of the conclusions reached on the basis of past work have too frequently been allowed to stand unquestioned and all subsequent observations have been interpreted in the light of these assumptions. In the accounts of the development of the vertebrate embryo such conditions seem to hold in respect to the nature of cell division. Judging from the description and from the lack of statements to the contrary, it appears to be accepted that at least the great majority of cell divisions are of the type known as mitosis or indirect cell division. Most of the deviations from this method that have been noted have been immediately assigned to that other obscure and little-understood process, amitosis.

Amitosis was at first considered to be the only method by which the cell divides. Later study led to the discovery of the more complicated process of mitosis. Following this, there developed a controversy concerning the meaning of amitosis and its place in the developmental processes of ani-



mals. Some considered it an important method of cell division, while others concluded that it was a process found only in senescent tissues, involving degenerating and dying cells which were in need of increased nuclear surface, and a process which never involved the division of the cytosome.

It is not the intention of the writer to enter into a lengthy account of this controversy or to cite the evidence offered in support of either side. For these details the reader is directed to the very complete account given by Wilson ('28) in his book, "The Cell in Development and Heredity," pages 214 to 223. The general conclusions reached, and those which seem to be rather generally accepted to-day, are that amitosis is a process of cell division of rare occurrence and of slight importance in the development of an organism and that it is found mainly in senescent tissue which has about run its course.

Various writers have mentioned and illustrated something other than mitosis in animals, but they have disposed of it as amitosis. Child ('07) reported amitosis in the chick embryo. What was designated amitosis may be seen in figures 86 and 87, of the striated muscle of the fish, in the book by Dahlgren and Kepner ('08), "Principles of Animal Histology." Conklin ('17) found in the egg of *Crepidula plana* a type of division which he spoke of as 'modified mitosis.' Shull ('26), in his book, "Heredity," states that in cases of amitosis found in the higher animals it is possible the process may be simply mitosis 'distorted beyond recognition.'

The writer has long been puzzled in the attempt to account for the rapid growth of the chick embryo through ordinary mitotic cell division. The number of mitoses present seems entirely inadequate to provide for the development of the various organs. During the last four years, attention has been directed toward the solution of this problem.

Cell division in the chick embryo was first investigated in developing striated muscle. Stages from those in which only mesenchyme was present to those of hatching were studied.

Following this, the other tissues of the chick were examined and the problem was attacked through all possible techniques of fixation and staining and through tissue culture. As a result of this study the writer has become convinced that mitosis as usually understood and figured plays very little part in the proliferation of the nuclei in the developing striated muscle and probably only a minor part in the development of the other tissues, and that it is replaced by a shortened and simplified process, which is not amitosis, but is probably a modified form of mitosis. In fact, no other explanation appears to explain the phenomena which have been observed. It seems most appropriate to employ for the type of nuclear division described in this paper the term used by Conklin, 'modified mitosis.'

#### MATERIALS AND METHODS

Chick embryos were obtained from eggs furnished by the University of Idaho Poultry Department. As a fixative unmodified Bouin fluid was used and all preparations were stained in Heidenhain's iron haematoxylin. I wish to express to M. M. Keith and S. E. Stoddard, former graduate students of the department, my sincerest thanks for the large series of preparations made available for study through their excellent technique and for their faithful and valuable assistance during the study of the first phases of this problem.

Of the illustrations, figures 1 to 32 are unretouched photomicrographs taken with a 90 $\times$  apochromatic objective and a 12.5 compensating ocular, while 33 to 60 are drawings. Figures 5 to 9 were made by Paul V. Woolley, Jr., a graduate student. Figures 14 to 24 are four-times enlargements from the original photomicrographic negatives.

#### OBSERVATIONS AND DISCUSSION

##### *I. Modified mitosis in developing striated muscle*

1. *Division of the chromatin.* Failure to find in the embryo enough mitoses to account for its rapid growth directed attention to the condition of the chromatin in the nuclei.

Intensive study revealed this substance to occur in a very high percentage of cases in the form of two rather conspicuous nucleoli. Sometimes there was only one of these, and at other times this might be seen to be dividing by elongation and constriction. This was what appeared upon first examination to be merely a separation of the chromatin into two approximately equal portions by a mass division. Superficially, it answered all the descriptions of amitosis and resembled the usual illustrations of this process (figs. 1, 2, 5 to 9, 14 to 23, 27, 28, 42 to 46, 56, 57).

In all the nuclei from the mesenchyme on through the stages of striated muscle formation to that of hatching, there appeared either one or two masses of chromatin (figs. 14 to 18). Dumbbell shapes (fig. 16) were found everywhere, with all stages between a slightly elongated primary mass (fig. 17) through slight and extreme dumbbell formations (figs. 14, 54, 56, 57) to conditions where the two daughter masses had just separated (figs. 14, 55, 60). Later these two masses would be found to have moved farther and farther apart (figs. 15, 19, 33 to 40, 48, 49, 59).

There is probably a rather rapid regeneration in size of these recently formed daughter masses. In many cases these chromatin masses were without evidence of definite structure (fig. 33), with surfaces simply more or less rough in character. However, in many, close observation brought out the presence of a knobby surface, as if the mass were the result of a partial fusion of a number of small bodies (figs. 4, 14, 15, 18, 22, 23, 24, 34). In very many cases this was so clearly evident that the masses may be designated 'mulberries.' It was often possible to make out this structure with unusual clearness and it could be seen that the outer part of the mulberry was covered with large, plainly appearing knobs which projected considerably from the surface. Further, one could sometimes see the outlines of the component parts quite clearly as light would penetrate between them (fig. 35). In a few cases there was sufficient spreading of these bodies that they could be recognized as discrete particles.

At the time of division there is no arrangement of these bodies on an equatorial plate as in mitotic division. The primary mulberry mass simply divides without *visible* division of its component bodies and the two daughter masses begin to draw apart exactly as in the figures illustrating amitosis. Lagging chromatin drawn out between the two masses can often be observed (fig. 56). Between the two daughter masses there can generally be made out a region staining less than the lagging chromatin, but more than the rest of the nucleus (figs. 8 and 14, *s*). More careful examination shows this region to contain fibers of exceeding fineness which can be recognized only with the greatest difficulty (figs. 34, 35, 38 to 40, 59). Rarely there may be made out what appears to be another group of fibers converging to a point from the outer edge of each chromatin mass. Between the two dividing masses of chromatin may be detected in all nuclei two round, intensely staining bodies, each surrounded by a clear spherical area (figs. 24 and 35, *a*). Staining shows these to be of chromatin.

The foregoing is merely a brief description of the phenomena as observed. There can be no question but that we are observing here a method of chromatin division which takes place within the nucleus. It has always been customary to interpret cytological phenomena through the establishing of a complete series, with all intermediate stages, covering the entire change in question. This series is most easy to demonstrate. This same thing has undoubtedly been seen many times, but has been dismissed as representing either amitosis or merely the presence of conspicuous nucleoli or karyosomes.

In attempting to interpret these phenomena it is necessary first to review the difference in definition between mitosis and amitosis. It seems to be the consensus of opinion that amitosis differs from mitosis principally in the fact that there is present in the former neither spireme, spindle, nor chromosomes, but that the nucleus and its chromatin divide by a simple mass division. Further, in mitosis, discrete chromo-

somes appear and arrange themselves upon a spindle, and centrosomes and asters are generally present. In other words, mitosis in the animal cell is characterized by the presence of: *a*) condensed, discrete chromosomes; *b*) spindle; *c*) centrosomes and asters. It has also been held by some that amitosis, unlike mitosis, is never followed by the division of the cytosome. The problem will be discussed under the above headings.

*a. Condensed, discrete chromosomes.* The chromatin found in the nuclei of developing striated muscle is largely concentrated in one or two masses, depending upon its stage of division. There is probably a rather rapid regeneration in the size of the recently formed daughter mulberries. Superficial examination of these masses, without a study of their activity, would immediately lay them aside as merely large karyosomes, or they might be called nucleoli. Their staining behavior easily identifies them as chromatin. A more careful examination, however, reveals the fact that they vary considerably in structure. As already described, some have merely a more or less roughened surface, while others have a surface resembling a mulberry, with definite, partly separate, rounded bodies. In abundant instances this configuration was very clear and unmistakable. Sometimes a looser association exists in which light passes through the mass and reveals definite bodies, and very rarely the latter appear spread out widely enough to make a count almost possible.

That there are separate bodies found in these chromatin mulberries is entirely certain; that these bodies are small, discrete chromosomes is highly probable. They may represent only the remnants of chromosomes. There is considerable evidence accumulating that at the inception of this type of division a rather large amount of chromatin is eliminated and comes to lie diffused throughout the nucleus, but especially around its periphery. Guyer ('16) states that the somatic number of chromosomes in the fowl is sixteen to eighteen. It has been impossible to obtain definite counts in these preparations, due to the small size of the bodies and to the great

degree of clumping which occurs in most cases, but from attempts at counts in the most favorable nuclei it can be seen that sixteen and eighteen are not impossible figures, but must lie close to the number present. Models may be made which will prove that the number cannot possibly be as low as six to eight, nor can it be as much greater than sixteen to eighteen, as, for example, twenty-five to forty. It is highly probable, therefore, that we are dealing with very small chromosomes in the regular somatic number. In most cases the fixation has exaggerated the clumping tendency so marked in avian and mammalian tissue, hence the densely contracted mulberries. But also it must be remembered the total amount of chromatin is very much less than that found in the nuclei undergoing typical mitosis.

If the small bodies present in the mulberries really are chromosomes, the method by which they divide (for we would expect this chromatin division to be a true qualitative and quantitative division) is still obscure. From all appearances the mulberry mass simply draws out, constricts, and then pulls apart exactly as in the process as described for amitosis, but it is very probable that each chromosome in this mass constricts or splits and then divides into two parts. The lagging chromatin so often seen between the mulberries is probably one or more chromosomes still undivided. That there occurs a division of each one of these chromosomes seems probable from the fact that in every one of the thousands of cases studied the division resulted in the production of two bodies of approximately equal bulk. Further, were each chromosome not to divide, we would expect to find a progressive decrease in the number of bodies in successive generations of mulberries until such a point would be reached when a count could easily be made. Such is not the case. Therefore the ability to distinguish what are probably chromosomes would seem to prove that there must be a qualitative and quantitative distribution of the chromatin to the two daughter masses. The acceptance of these bodies as chromosomes would preclude this process being interpreted as amitotic.

b. Spindle. During the drawing apart of the two masses of chromatin there appears between them a band of material in which it is possible under critical optical conditions to make out exceedingly fine fibers (figs. 8 and 14, s, and 34, 35, 38 to 40, 59). This band can be distinguished from lagging chromatin by the difference in staining. Whether these fibers are really spindle fibers is uncertain, but in all probability they represent an attempt at their production. This would constitute a second argument against this process being amitotic.

c. Centrosomes and asters. Centrosomes and asters have never been demonstrated in this material. It is true that in a few cases the very fine fibers converging from the recently divided mulberries *seemed* to end in a very minute body, still the size of this is too small (if it exists at all) to be considered a centrosome. In addition, it must be held in mind that during the process of chromatin division being described the nuclear membrane at no time disintegrates so as to allow the nuclear contents to lie in the cytoplasm as in mitosis, and hence centrosomes if present would have to be of the intranuclear variety hitherto reported only rarely. Also, we would have present in the same tissue centrosomes in the cytoplasm in mitotic cells and the same organ within the nucleus in modified mitotic cells. Faulty technique cannot be blamed for failure to demonstrate the centrosomes, as these appeared with the greatest clearness in all cases of typical mitosis in the same preparations. Therefore the conclusion seems to be justified that centrosomes do not appear in this type of division.

Since the embryonic tissues of the chick are highly syncytial in character, the question of whether division of the cytosome does or does not follow this division of the nucleus need not be considered; this phenomenon is one of the nucleus only.

2. *Division of the nucleus.* The nuclei of the premuscle mesenchyme are circular or slightly oval in outline. As soon as the transition to striated muscle has taken place and the

nuclei lie within the fibers, they elongate, assuming a length of about twice their diameter. In the various stages of developing muscle, especially from ten days to hatching, nuclei of two to three times normal length are common (figs. 2, 8, 10, 13, 42 to 44).

Division of the chromatin, as has already been described, is in all cases followed by division of the nucleus. This is always brought about in exactly the same fashion throughout the entire series of stages. A partition or 'nuclear plate' is formed across the nucleus, dividing it into two parts (figs. 19 and 20, *a*). Then the nucleus divides along this plate. The plate may be formed at right angles or it may be diagonal (fig. 41). It can scarcely be identified until about the time it is fully formed, when it becomes definitely recognizable. Separation probably takes place quite soon after this plate has formed. Only in the rarest cases were nuclei found to constrict dumbbell fashion, and even then, division was actually accomplished by nuclear-plate formation. No cases of complete division by constriction alone were ever observed.

Soon after the nuclear plate forms, there comes about a separation of the two daughter nuclei. Their ends, once contiguous (figs. 38, 41, 58), draw apart, at first retaining the shape of the plate (figs. 41, 52, 53), then rounding up (fig. 39). For a time their ends still touch; then they draw farther apart (fig. 40). Many nuclei could be recognized as sister nuclei, especially in cases where the nuclear plate had been very irregular; here the two withdrawing nuclei still retained their shape and their ends still conformed with each other (figs. 21, 22, 52, 53). Later these would round up.

3. *Precocious division of the chromatin.* The striated muscle during the later stages of the incubation of the chick was characterized by the peculiar character of some of the nuclei. First, there were nuclei present which were several times more than average length. Secondly, there were found long strings of nuclei of normal length, from three to as many as fifteen in number, closely packed end to end (figs. 11, 12, 41, 58). These latter are to all appearances due to the



rapid division of the abnormally long nuclei into a number of daughter nuclei and the subsequent division of the latter.

Very frequently the chromatin is found to divide more than once before the nucleus itself divides. Commonly one finds three masses of chromatin in a nucleus. This is due to the redivision of one of the daughter mulberries. In the long nuclei as many as five or six clumps of chromatin may be present. Following this, as many nuclear plates are formed as are necessary to segregate the separate chromatin masses. For a time these resulting nuclei remain close together; then they draw apart and the ends round up. The extent to which this method is used in striated muscle has not yet been determined, but it is probably a means of nuclear proliferation correlated with the rapid elongation of the muscle fibers.

## *II. Modified mitosis in other tissues of the chick*

In addition to the study of modified mitosis in striated muscle, all of the other tissues of the chick embryo were investigated. Modified mitosis has been found in the mesenchyme (figs. 1, 5, 9), amnion, ectoderm (fig. 3), entoderm (fig. 4), brain and spinal cord (fig. 28), gut (fig. 31), liver (fig. 30), mesonephros (fig. 26), heart, cartilage (fig. 27), and skin (fig. 32). In all these tissues it is essentially the same process as has already been described. However, there is considerable variation in the total amount or bulk of the chromatin found in these various kinds of tissues. In some the mulberries are quite small in comparison with those in striated muscle. The nuclei of the central nervous organs seem to be especially rich in chromatin. But in no case does the amount of this substance approach that found in nuclei undergoing typical mitosis.

The division of the nucleus in these various tissue cells is brought about by nuclear-plate formation much as was found in striated muscle, but no long nuclei exist outside of the latter tissue.

## CONCLUSIONS

There can be no serious question in the mind of anyone who has devoted sufficient effort to the material such as the writer has studied as to the fact that there is found here a method of nuclear division and that it is not typical mitosis. It is impossible to break the process up into the usual 'phases' such as are used in describing mitosis. No prophase with a spireme is present, and if there is a resting stage, it consists merely of that period when the nucleus contains only one mulberry. An attempt has been made to show on the basis of the highly probable presence of discrete chromosomes and of spindle fibers that this is not amitosis, but more akin to mitosis. The absence of centrosomes and asters offers no great difficulty, as they are not necessary for the division of the nucleus of the higher plants and their presence within the nucleus in the chick cell would be very hard to explain. As long as we are dealing with highly syncytial tissue, the question of the division of the cytosome is not a consideration.

The process of modified mitosis may be looked upon as a modification of the mitotic process brought about largely as a concession to the speed required in a rapidly growing embryo. Such rapid extension in length as takes place in the formation of the embryo's muscles accounts for the peculiar methods of nuclear division observed. To produce a hatched chick in twenty-one days calls for tremendous cell activity and nuclear proliferation, with consequent chromatin division. Though there are as yet no data available on the time required for one of these divisions to take place, it may well be, on account of its simpler form, somewhat less than that required for mitosis proper.

Exactly what relation exists between the chromatin of the nucleus and the cytoplasm of somatic cells is far from being understood. Whether the full complement of chromatin, either as to number of chromosomes or total amount of the substance, is a necessity for each somatic cell is not known. In this case there is considerable evidence that a certain

amount of the original chromatin is not used in the production of the mulberry masses when this method of division begins and that this lost chromatin is thus probably in time largely dissipated. It is also very evident that the amount of chromatin present in nuclei undergoing modified mitosis is a mere fraction of the amount one may see in some near-by nucleus which is in the process of mitosis (figs. 5, 9, 13, 31, 48, 49). On the basis of this process being essentially mitotic, and the division of the chromatin being qualitative and quantitative, it would seem that some tissues, at least in the embryo, might not need the total amount of chromatin, as seen in full-sized chromosomes, in order to carry on their activities.

Proving this process to be essentially mitotic would also remove the objection always voiced against the idea that amitosis was a real process of cell division, namely, that it was contrary to the chromosome theory of heredity. If it is not mitosis, it is amitosis, and here we have it not in senescent tissue, but in the most active tissue known—embryonic. Since it is certain that not only striated muscle, but also most of the other tissues of the chick embryo owe their nuclear proliferation to the process described, a method as rough as amitosis and so entirely hostile to the very principles of heredity could scarcely be accepted as responsible for so large a percentage of the task of forming an animal.

The writer does not feel capable of passing upon the reports in the past of amitosis, but the conviction has been growing that very possibly some portion of these cases really may have been some modified form of mitosis. Nor does he feel it safe at this time to extend this process to cover the cell divisions in any other groups of animals. Work has been started on this and it is hoped that it may be cleared up at a later time.

#### SUMMARY

1. In the nuclei of all stages of developing striated muscle of the chick embryo the chromatin is found in masses, generally one or two in number.

2. Some of these masses have only slightly roughened surfaces, while others are covered with round protuberances and are designated 'mulberries.'

3. In some cases these mulberries can be seen to be formed of a clump of small, round bodies; in rare cases these are separated enough to be almost countable.

4. These bodies are present, in as far as their number can be estimated, in approximately the same number as the somatic number for the fowl.

5. These mulberries divide into two daughter masses by elongation, dumbbell formation, and constriction.

6. There is no progressive diminution in the apparent number of these bodies found in the mulberries resulting from successive divisions.

7. The two daughter mulberries are, without exception, of approximately equal size.

8. These bodies are very probably small chromosomes, closely clumped through fixation, and each one probably divides at the division of the mulberry.

9. Exceedingly delicate fibers, possibly representing an attempt at a spindle, are present.

10. Neither centrosomes nor asters are present.

11. These phenomena all occur within the non-disintegrating nuclear membrane, and, striated muscle being syncytial, the question of the subsequent division of the cytosome need not be considered.

12. The nuclei divide by formation of a nuclear plate; they rarely show constriction and never divide by the latter method alone.

13. Extremely long nuclei are found in the later stages of incubation. These show precocious division of the chromatin, then division by a number of nuclear plates, producing long rows of contiguous, sister nuclei.

14. The term 'modified mitosis' is used for this process on the basis of the probable presence of chromosomes and spindle; the absence of centrosomes and asters is immaterial.

15. This is almost the exclusive method of nuclear division found in the striated muscle of the chick embryo.

16. Essentially the same method of division has been found in practically all of the other tissues of the chick embryo.

17. This method of nuclear division may possibly be a concession to the necessity for speed in the production of a rapidly growing embryo.

18. This method of nuclear division, in contrast to amitosis, harmonizes with the chromosome theory of heredity.

19. Modified mitosis is probably responsible for the greater percentage of nuclear divisions in all of the principal tissues of the chick embryo.

#### LITERATURE CITED

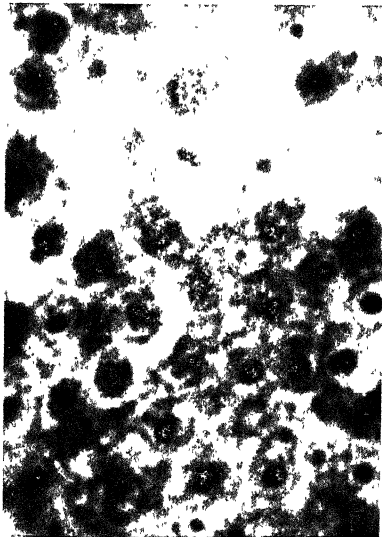
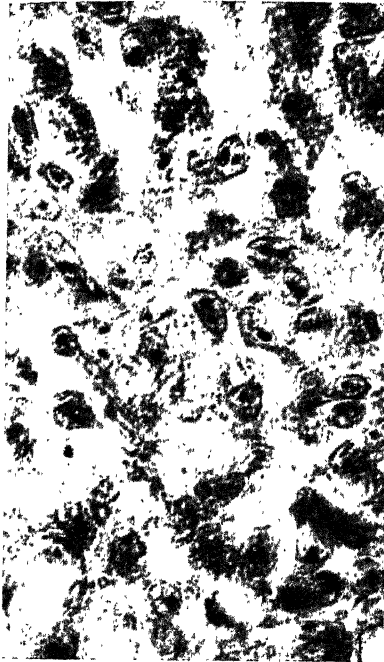
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## PLATES

## PLATE 1

### EXPLANATION OF FIGURES

- 1 Mesenchyme from 120-hour chick.  $\times 1000$ .
- 2 Striated muscle from 450-hour chick.  $\times 1000$ .
- 3 Ectoderm from 35-hour chick.  $\times 1000$ .
- 4 Entoderm from 35-hour chick.  $\times 1000$ .

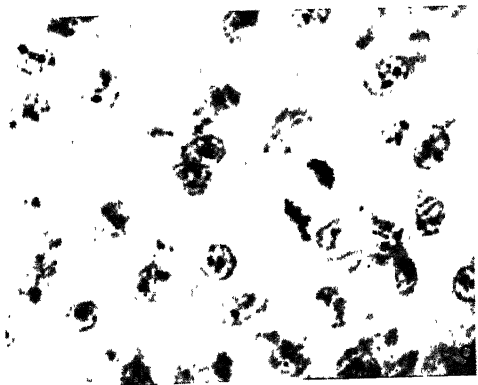
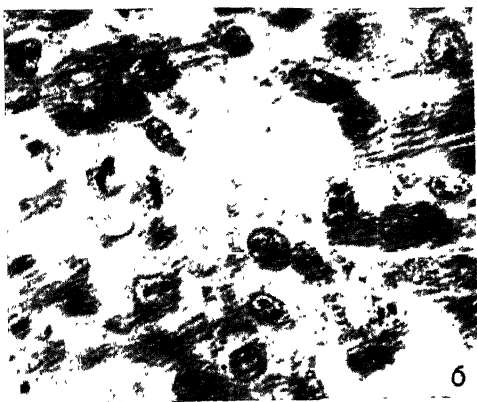




## PLATE 2

### EXPLANATION OF FIGURES

- 5 Mesenchyme from 96-hour chick.  $\times 1000$ .
- 6 Developing striated muscle from 146-hour chick.  $\times 1000$ .
- 7 Same, 192-hour chick.  $\times 1000$ .
- 8 Same, 192-hour chick. Spindle fibers (?) at *s*.  $\times 1000$ .
- 9 Mesenchyme from 96-hour chick.  $\times 1000$ .



### PLATE 3

#### EXPLANATION OF FIGURES

- 10 Striated muscle from 236-hour chick. Nuclear plate, *a*.  $\times 1000$ .
- 11 Striated muscle from 421-hour chick, showing string of nuclei. Nuclear plates may be seen forming between the chromatin masses.  $\times 1000$ .
- 12 Same from 237-hour chick.  $\times 1000$ .
- 13 Striated muscle from 237-hour chick. Two nuclei appear in anaphase of mitosis. This figure illustrates the difference in the amount of chromatin in the nuclei undergoing these two kinds of division.  $\times 1000$ .



## PLATE 4

### EXPLANATION OF FIGURES

All figures in this plate have been enlarged four times.

14 Two nuclei from mesenchyme of 120-hour chick, showing two stages of division of mulberry. Spindle fibers (?) at *s*.  $\times 1000$ .

15 Same from cartilage of hmb of 187-hour chick.  $\times 1000$ .

16 Same. One nucleus shows an undivided mulberry, while the other contains a dumb-bell.  $\times 1000$ .

17 Same. Mulberry elongating.  $\times 1000$ .

18 Same. Mulberry just divided.  $\times 1000$ .

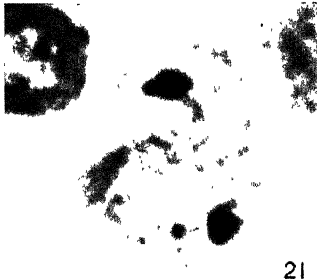
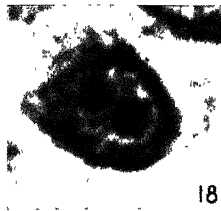
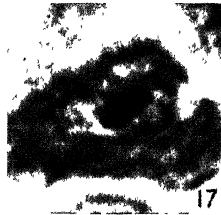
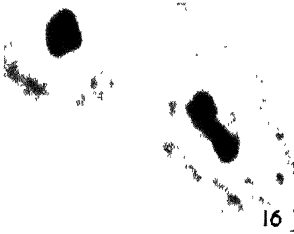
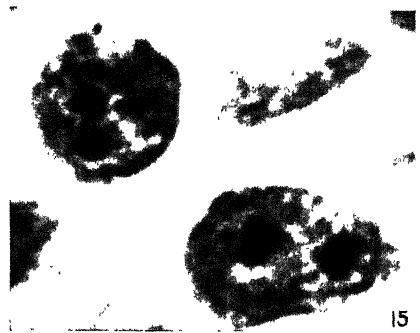
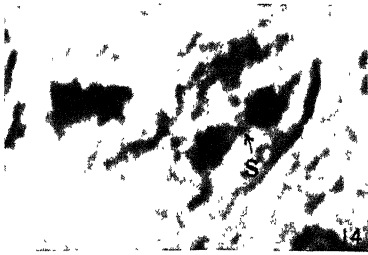
19 Same. Nuclear plates, *a*, forming across each nucleus.  $\times 1000$ .

20 Nuclei from 120-hour chick mesenchyme, showing nuclear plates, *a*, forming.  $\times 1000$ .

21 Same from cartilage of 187-hour chick.  $\times 1000$ .

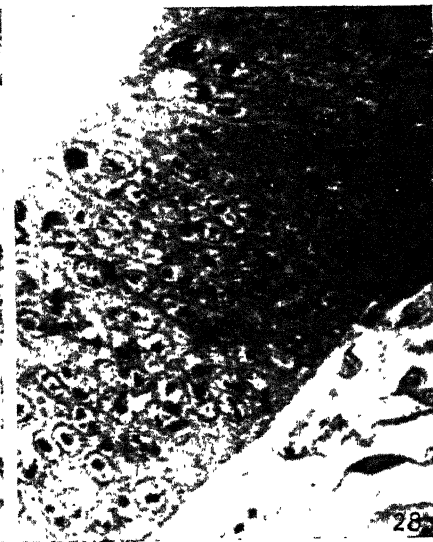
22 Same from 120-hour chick mesenchyme.  $\times 1000$ .

23 Same, showing two nuclei which have just separated following nuclear-plate formation.  $\times 1000$ .





24 Developing striated muscle from 192-hour chick. Shows two dumb-bells. At right of figure appears a nucleus with two small chromatin bodies, *a*, between the two mulberries.  $\times 1000$ , enlarged four times.



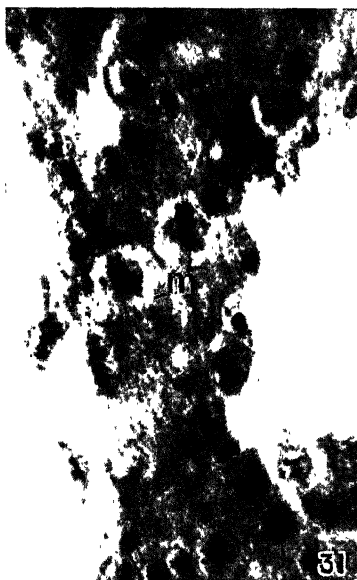
- 25 Notochord from 80-hour chick.  $\times 1000$ .  
26 Mesonephros from 237-hour chick.  $\times 1000$ .  
27 Cartilage from limb of 187-hour chick.  $\times 1000$ .  
28 Central nervous system from 104-hour chick.  $\times 1000$ .



## PLATE 7

### EXPLANATION OF FIGURES

- 29 Developing striated muscle from 239-hour chick, showing string of three nuclei, *n*.  $\times 1000$ .
- 30 Liver from 237-hour chick.  $\times 1000$ .
- 31 Digestive system from 237-hour chick. Two mitoses, *m*.  $\times 1000$ .
- 32 Epidermis, *e*, forming dermis, *d*, from 239-hour chick.  $\times 1000$ .

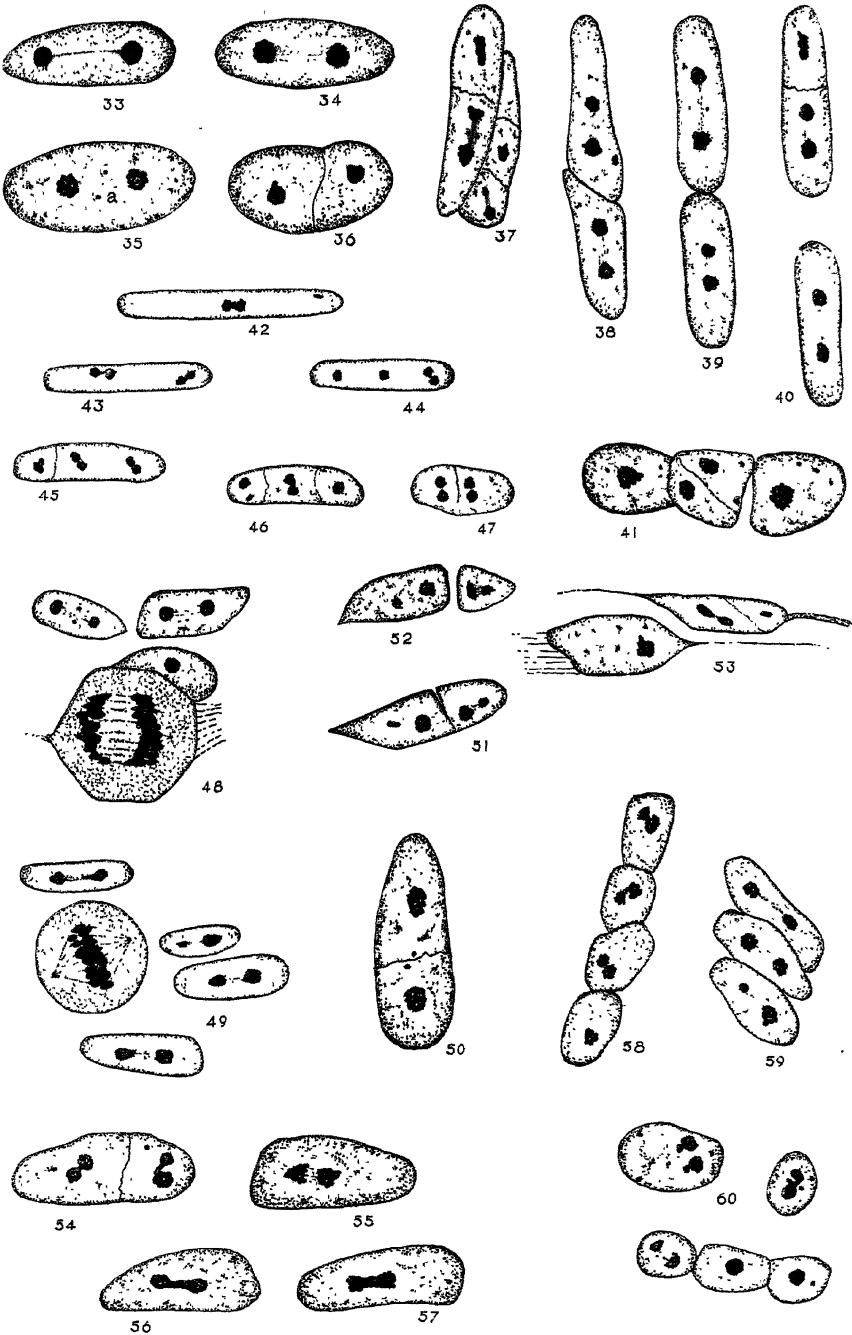


## PLATE 8

### EXPLANATION OF FIGURES

Figures 33 to 60 represent nuclei only from striated muscle and are from drawings.

- 33 Chromatin masses showing no visible structure. 257 hours.
- 34 Same, in form of 'mulberries.' 257 hours.
- 35 Same, showing indications of presence of small, discrete bodies. Two small chromosomes (?) at *a*. 257 hours.
- 36 Nuclear plate. 257 hours.
- 37 Precocious division of chromatin. Three nuclear plates. 400 hours.
- 38 Two nuclei with ends still in close contact. 412 hours.
- 39 Same, with ends rounded up. 412 hours.
- 40 Same, after withdrawal. Upper one is again dividing. 412 hours.
- 41 String of three related nuclei. 227 hours.
- 42 Long narrow nuclei from well-differentiated muscle. 492 hours.
- 43 to 47 Same, showing precocious division of chromatin and formation of nuclear plates. 492 hours.
- 48 Typical and modified mitosis. 257 hours.
- 49 Same. 257 hours.
- 50 Nuclear plate. 227 hours.
- 51 Nuclei about to separate along transverse nuclear plate. 403 hours.
- 52 Nuclei recently separated as in figure 51. 403 hours.
- 53 Nuclei which have separated along an oblique nuclear plate. 403 hours.
- 54 Precocious division of chromatin. 227 hours.
- 55 Chromatin masses slightly separated. 227 hours.
- 56 Chromatin masses pulling out into dumb-bells. 227 hours.
- 57 Same. 227 hours.
- 58 String of related nuclei. Chromatin masses in division. 227 hours.
- 59 Same. Preparation probably along oblique plates. 227 hours.
- 60 Nuclei from 257-hour stage.





# DEVELOPMENTAL STAGES OF THE CHONDROCRANIUM IN SOME SELACHIANS

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## FIFTEEN FIGURES

### AUTHOR'S ABSTRACT

Serial sections of seven embryos were examined, in four of which the chondrocrania were reconstructed. Parachordals appear as independent precartilages on each side of the notochord in the otic region. Later chondrification occurs, and they fuse with the notochord. Orbitals and trabeculae appear as independent precartilages; later, as cartilages they fuse with the basal plate and with each other, forming the cranial wall between the orbital cavity and brain. An apparent shifting posteriorly of the bases of the fifth, seventh, and eighth cranial nerves, in respect to the anterior end of the notochord and to the otic vesicles, occurs between the 10-mm. and the 60-mm. stages. The otic vesicles, proportionately small in the 10-mm. stage, reach their relatively largest size in the 19-mm. embryo. The floors of the otic capsules begin as lateral extensions of the basal plate at the 25-mm. stage, and from these anlagen chondrifications extend, forming the anterior and posterior cupolas. Most of the tegmen cranii forms by chondrifications that approach the median plane from each side. It is practically complete in the 60-mm. embryo. The cervical neural arches also contribute in forming the tegmen cranii and the occipital region. The ventrally recurved end of the notochord of the 10-mm. stage persists through the 60-mm. embryo.

A full understanding of the vertebrate skull is far from being complete. Any investigation on this subject, which contributes in clearing up even some of the minor details, may possess far-reaching results. A better insight into the development of those low-typed vertebrates suggests a possible key to the solution of the problem. These forms selected for investigation occupy a unique position in relation to all chordates, for they are among the simplest of the gnathostomes and are also the representatives of the oldest living vertebrates. While a better knowledge of the chondrocranial development of these forms may be of no particular importance within itself, yet any information gained from them throws light on the interpretation of homologies and the ancestry of higher forms.

## REVIEW OF LITERATURE

The literature on the subject, while not extensive, is difficult to follow, because in many cases the same structure has been designated by several different names. This condition came about frequently, because names of parts were first applied to higher forms, and then structures of somewhat similar shape and position were given the same name, often without investigating the ontogenetic or phylogenetic development. Parker ('79) presented a classic on the structure and development of the skull in the sharks and skates, using such forms as *Scyllium*, *Pristiurus*, and *Raia* for the basis of his investigation, mentioning *Squalus acanthias* only incidentally. Sewertzoff ('99) presented a very good paper on the development of the selachian skull, showing figures of representative stages, but the writer has been able to examine younger specimens than he considered. Gaupp ('06) brought together all the more important references on the development of the vertebrate and gave extracts and figures of the outstanding contributions on the development of the selachian chondrocranium. Wells ('17) gave an accurate description of the chondrocranium of the adult *S. acanthias* under the less familiar name of *Acanthias vulgaris*. Daniels ('22) brought together the evidence of the more important papers on various adult elasmobranch skulls, but included very little on their development. van Wijhe ('22) made a worthy contribution to our knowledge of the development of the skull in *Squalus acanthias* under the older name of *Acanthias vulgaris*, giving particular attention to the early stages, but considers none larger than 39.5 mm. de Beer ('26) discussed at length the orbitotemporal region of the vertebrate skull, but mentions *Squalus acanthias* only incidentally and that is of the adult form. Goodrich ('30) presented an excellent review of the literature on the development of the vertebrate skull, but gives little attention to *Squalus acanthias*.

## MATERIALS AND METHODS

The specimens used in this investigation were the following stages of *Squalus acanthias*: 10 mm., 19 mm., 25 mm., 27 mm., 35 mm., and 60 mm.; also one 40 mm., of *Squalus sucklii*; measurements being made from tip of nose to end of the tail. Models were made of the 25-mm., 35-mm., 40-mm., and 60-mm. stages. No special technique seemed necessary in order to get desirable microscopic sections. Some of the specimens were killed and fixed in 3 per cent formalin, others in 70 per cent alcohol, and still others in Bouin's. The material was passed through the various grades of alcohols in the usual way and cleared in xylol. The series were embedded in paraffin, cut 15 to 30  $\mu$  thick, and stained in iron hematoxylin. Two methods were used in reconstructing the models: some were made of beeswax plates according to the well-known Born method, others were made of a standard grade of straw-board—a method devised by Harrison ('24).

## NOTOCHORD AND PARACHORDALS

The youngest *S. acanthias* investigated was a 10-mm. embryo, and little of the chondrocranium is present at this stage besides the notochord. Careful measurements were made of the diameter and circumference of the notochord by means of the camera lucida, and from the data obtained the outline drawing (fig. 1) was constructed. The notochord gradually tapers cranially, and varies slightly in different regions. The notochord possesses at its anterior extremity a recurved tapering ventral hook, which extends backward 50  $\mu$ . A transection taken approximately at right angles to the axis of the embryo (fig. 2) shows the recurved notochord, and another 25  $\mu$  farther posterior (fig. 3) shows the notochord cut in two places. The latter section shows the condition near the extreme tip, where only three cells appear. The recurved portion thus decreases in size rapidly, and ends in a sharp point. Scammon ('11) shows an embryo 11.5 mm. long, which has a ventrally recurved notochord practically the same as the author's 10-mm. stage. This flexure of the



## ABBREVIATIONS FOR ALL FIGURES

<i>AC.</i> , antorbital crest	<i>OL.</i> , olfactory capsule
<i>AF.</i> , anterior fontanelle	<i>OP.</i> , otic placode
<i>AORS.</i> , anterior orbital shelf	<i>OPL.</i> , optic pedicle
<i>BPL.</i> , basal plate	<i>ORB.</i> , orbital
<i>DE.</i> , endolymphatic duct	<i>OS.</i> , foramina for ophthalmicus superfacialis
<i>DI.</i> , diencephalon	<i>OV.</i> , otic vesicle
<i>EP.</i> , ethmoid plate	<i>PC.</i> , precartilage
<i>FB.</i> , foramen for blood vessels	<i>PF.</i> , pineal foramen
<i>FBR.</i> , forebrain	<i>POP.</i> , postorbital process
<i>FEND.</i> , foramen for endolymphatic duct	<i>POT.</i> , postotic process
<i>FH.</i> , fenestra hypophyseos	<i>PP.</i> , parachordal plate
<i>FM.</i> , foramen magnum	<i>PQ.</i> , pterygoquadrate
<i>HA.</i> , hyoid arch	<i>PQP.</i> , pterygoquadrate process
<i>HBR.</i> , hindbrain	<i>RAP.</i> , Rathke's pocket
<i>HM.</i> , hyomandibular cartilage	<i>RB.</i> , rostral bar
<i>INF.</i> , infundibulum	<i>RP.</i> , rostral plate
<i>IW.</i> , inner wall of otic capsule	<i>SORC.</i> , supra-orbital crest
<i>LC.</i> , labial cartilage	<i>STM.</i> , synotic tectum
<i>MC.</i> , Meckel's cartilage	<i>TCE.</i> , tegmen cranii
<i>NO.</i> , notochord	<i>TE.</i> , trabecula
<i>OC.</i> , otic capsule	<i>TRC.</i> , transbasal canal
<i>ODE.</i> , opening of ductus endolymphaticus	<i>VA.</i> , visceral arches
	<i>I-X.</i> , foramina for cranial nerves

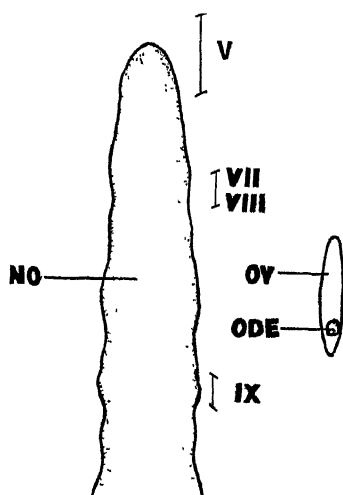


Fig. 1 Dorsal view of the notochord, otic vesicles, and the endolymphatic duct of a 10-mm. *Squalus acanthias*. Constructed from camera-lucida measurements.  $\times 62\frac{1}{2}$ .

notochord is correlated with that of the brain, and undoubtedly the enormous growth of the latter brings about the condition in the former. In the slightly oblique section of the 10-mm. specimen (fig. 4) the first indication of the parachordals is represented by prechordal cells below the otic vesicles, and separated from the notochord by a distance equal to one-half of its diameter in this region. At this stage these pre-

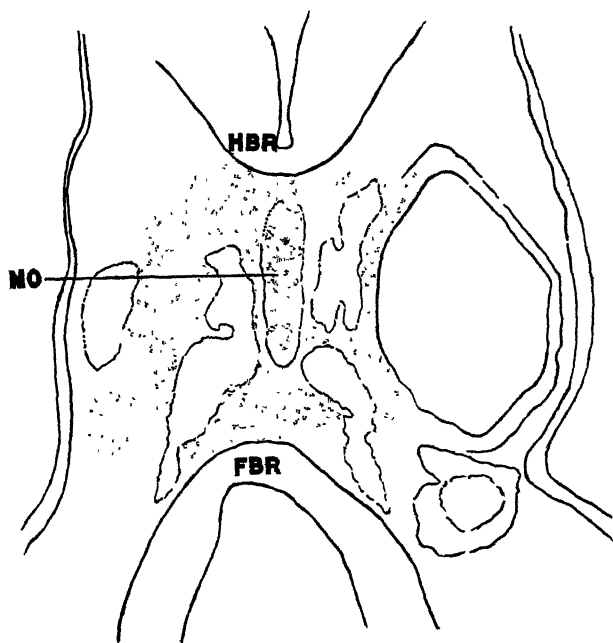


Fig. 2 Transection taken through the recurved anterior portion of the notochord of a 10-mm. *S. acanthias*.  $\times 62\frac{1}{2}$ .

chordal cells are separated on each side from the notochord through their entire areas. In the 10-mm. and also in the 19-mm. embryo, the anterior end of the notochord in front of the parachordals tapers much more gradually than Sewertzoff ('99) shows. There is a distinct recurved hook at the anterior end of the notochord which Sewertzoff fails to show. It was found, as did he, that the first anlage of the skeleton arises as two strips of precartilage lying on each side of the notochord, but the writer finds no lateral processes indicat-

ing the auditory capsule, nor the trabeculae, both of which were found by Sewertzoff. Therefore, it would seem that the writer's 19-mm. embryo, as well as his 10-mm. embryo, is younger than the youngest figured and described by Sewertzoff.

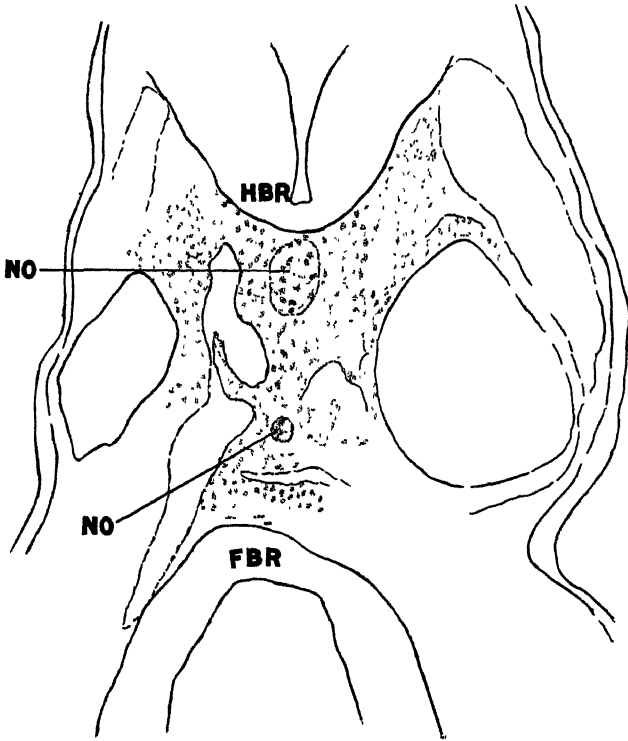


Fig. 3 Transection taken  $25\mu$  posterior to figure 2, showing the notochord cut in two places; 10-mm. *S. acanthias*.  $\times 62\frac{1}{2}$ .

In the 25-mm. stage the notochord is cylindrical and of about the same diameter as in the 19-mm. embryo as far forward as the anterior end of the otic vesicle. When viewed from the dorsal surface (fig. 5), the notochord tapers at first gradually, then more abruptly to the tip, which is bent downward and backward as in the 10- and 19-mm. embryos. The parachordal chondrification areas are now observed to have firmly united on either side with the notochord. Figure 6 represents a median vertical section through the ventrally

recurved hook of the notochord at the 25-mm. stage. With the exception of the recurved portion, the notochord is not cut through its diameter. Hence, its exact dimensions posterior to the hook are not shown. It will be observed that the ventral tip of the notochord gradually merges with the mesenchyme, in such a way that it is practically impossible to dis-

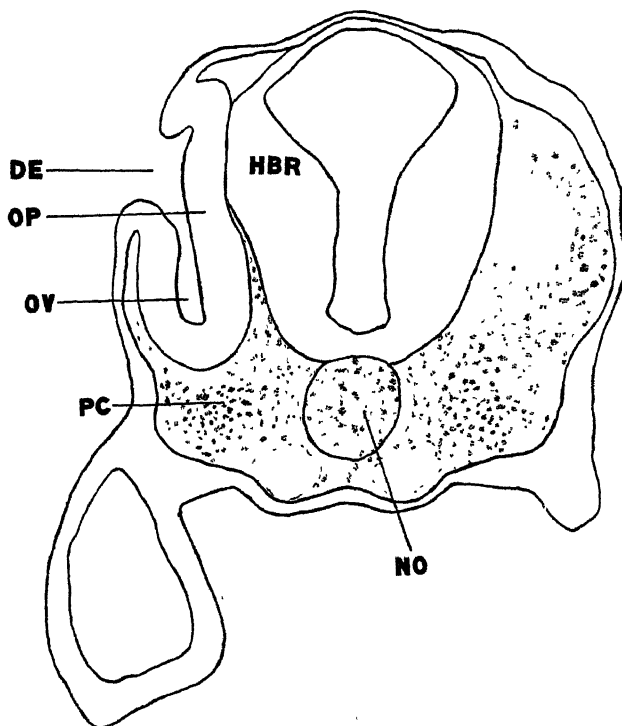


Fig. 4 A slightly oblique section through the otic region on one side and posterior to it on the other, showing relations of hindbrain, notochord, otic vesicle, and anlage of its capsule; 10-mm. *S. acanthias*.  $\times 62\frac{1}{2}$ .

tinguish its exact limits. Furthermore, the close proximity, of the medulla above and the infundibulum below, to the recurved hook are clearly shown. The anterior curved end of the notochord is much smaller proportionately than the remainder of it in the head.

In the 35-mm. embryo (figs. 7, 8, and 9) the basal plate has become broader, thicker, and the parachordals have extended

farther forward. The posterior ends of the alisphenoids have united with their anterolateral edges. The ventrally recurved anterior hook of the notochord persists, but is smaller, in proportion to its other transections, than in the younger stages. It appears as though the main portion of the notochord has continued to develop, while the recurved

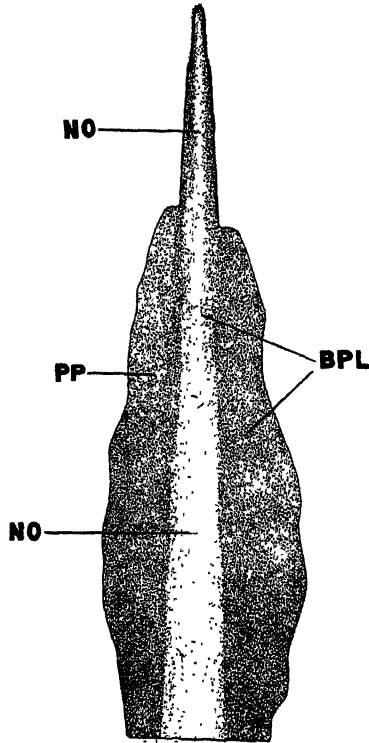


Fig. 5 Dorsal view of the reconstructed basal plate, as formed by the fusion of the parachordals with the notochord; 25-mm. *S. acanthias*.  $\times 33\frac{1}{2}$ .

portion has remained at approximately the same size. This 35-mm. stage is intermediate between the stages indicated in figures 5 and 13 by Sewertzoff ('99), but it is impossible for the writer to determine the limits of the parachordal plates in his figure 5. From the material at hand it is found that the lateral limits of the parachordals are more definitely determined than he gives them, or as they are found

in the earlier stages. The parachordals are contributing to the formation of the otic capsules (fig. 7), and as they become so intimately associated with the orbitals and trabeculae in the more advanced embryos, further discussion of them for this stage will be included subsequently under that heading.

Although a different species, the 40-mm. *S. sucklii* presents (figs. 10, 11, and 12) many conditions which are intermediate

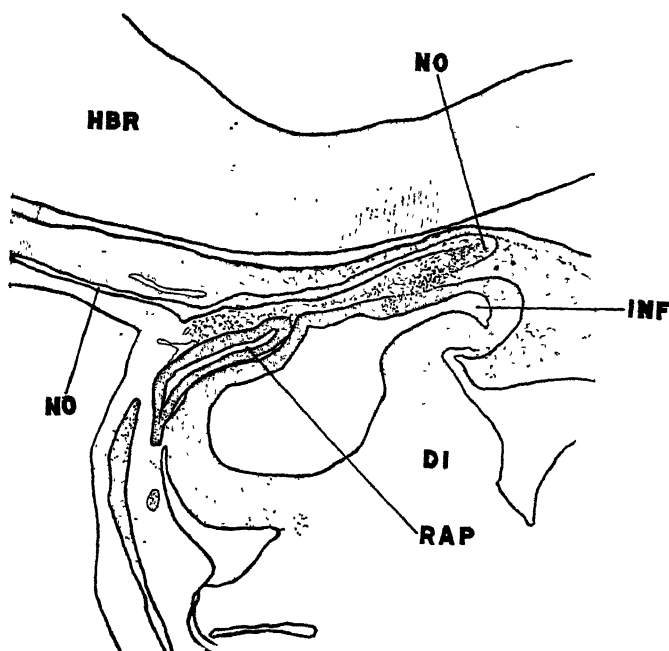


Fig. 6 Median vertical section, showing recurved anterior end of the notochord, with its relation to the infundibulum, Rathke's pocket, and brain vesicles; 25-mm. *S. acanthias*.  $\times 95$ .

between the 35-mm. and 60-mm. stages of *S. acanthias*. Its developing chondrocranium is much heavier and more definitely formed than in the previous stages. The basal plate (fig. 10) extends farther forward and is more dense than in the preceding specimen. The parachordals unite with the notochord on each side over more of its surface. Precartilaginous cells are differentiating in the otic region and migrating toward the center of the embryo, both above and below the notochord.

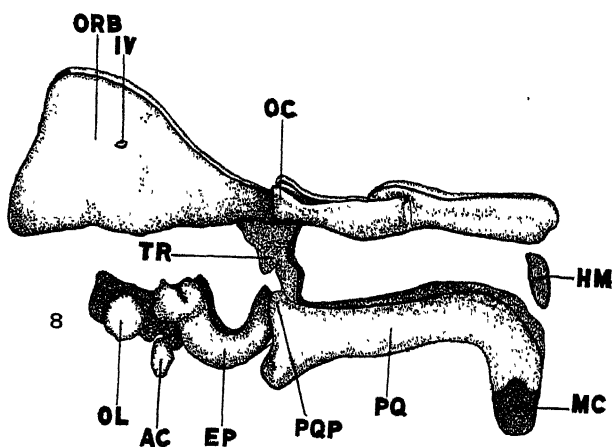
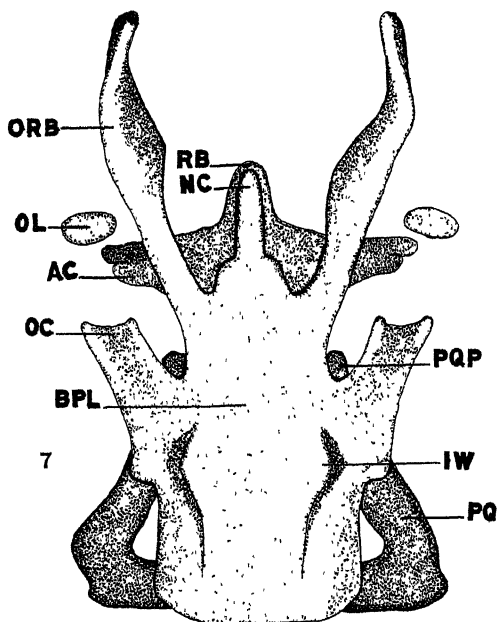


Fig. 7 Dorsal view of the reconstructed chondrocranium, showing the basal plate, otic capsule, and orbitals; 35-mm. *S. acanthias*.  $\times 16\frac{1}{2}$ .

Fig. 8 Left lateral view of the reconstructed chondrocranium, showing relations of the orbitals, trabeculae, ethmoid plate, and mandibular arch; 35-mm. *S. acanthias*.  $\times 16\frac{1}{2}$ .

The degree of advancement in this particular is most marked in the posterior otic region, and gradually decreases in extent farther forward, becoming scarcely noticeable in the anterior region of the parachordals. The ventrally recurved hook is retained by the notochord, which appears much like that in the younger stages of *S. acanthias*. Further relations of the basal plate to its adjacent structures are given below under their respective headings.

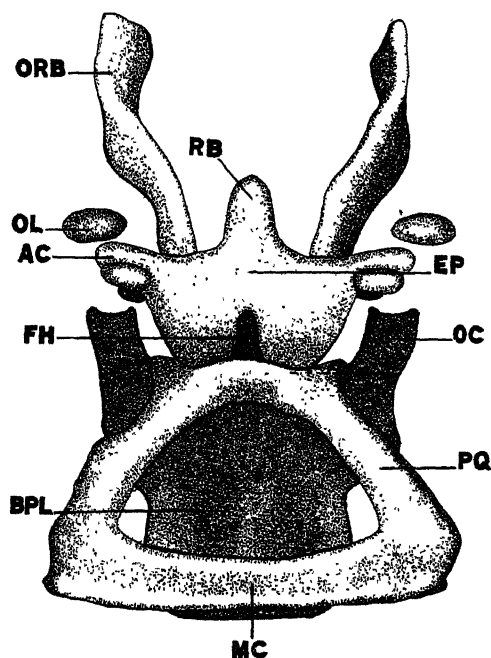


Fig. 9 Ventral view of the reconstructed chondrocranium, showing the ethmoid plate, fenestra hypophyseos, pterygoquadrate, and Meckel's cartilage; 35-mm. *S. acanthias*.  $\times 16\frac{1}{2}$ .

#### OTIC REGION

The relative sizes and positions of the otic vesicle and endolymphatic duct, in respect to one another and to the anterior end of the notochord, in the 10-mm. embryo (fig. 1), are worth considering. It is observed that the otic vesicle is only slightly longer than the diameter of the notochord in the same region; the endolymphatic duct opens on the dorsolateral



surface of the head over the posterior region of the vesicle. Scammon ('11) shows a figure of a 9-mm. embryo, where the endolymphatic duct opens externally at approximately the same position as the writer finds it. However, in his 11.5-mm. specimen this duct is shown opening posterior to the origin of the glossopharyngeal nerve. The relative position of the invaginating otic vesicle to the notochord and to the

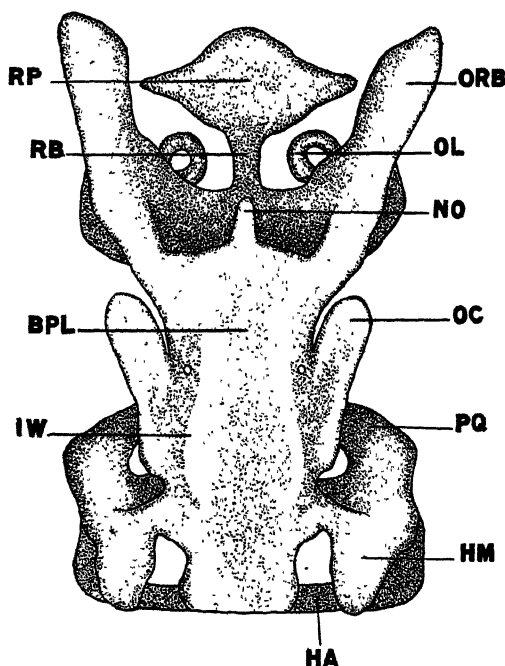


Fig. 10 Dorsal view of the reconstructed chondrocranium, showing relations of the rostral plate, orbitals, and olfactory capsules; 40-mm. *S. sucklii*.  $\times 16\frac{1}{2}$ .

fifth, seventh, eighth, and ninth cranial nerves is shown (fig. 1). It is especially interesting to note how far forward the base of the trigeminal nerve is located; also, that the otic vesicle lies wholly posterior to the region of the facialis nerve. The section taken obliquely through the 10-mm. embryo (fig. 4) passes through the center of the external opening of the endolymphatic duct on one side and behind the duct and vesicle on the other. The otic vesicle appears relatively deep,

with greatly thickened ectodermal lining and a large external opening of the endolymphatic duct. As has already been noted, the precartilaginous cells, the anlage of the parachordal plate, are localizing in the region ventral to the otic vesicle and lateral to, but not connected with, the notochord.

In the 19-mm. embryo the otic vesicles extend on each side dorsal to and along the cephalic half of the parachordals. The anterior limits of the otic vesicle extend in front of the

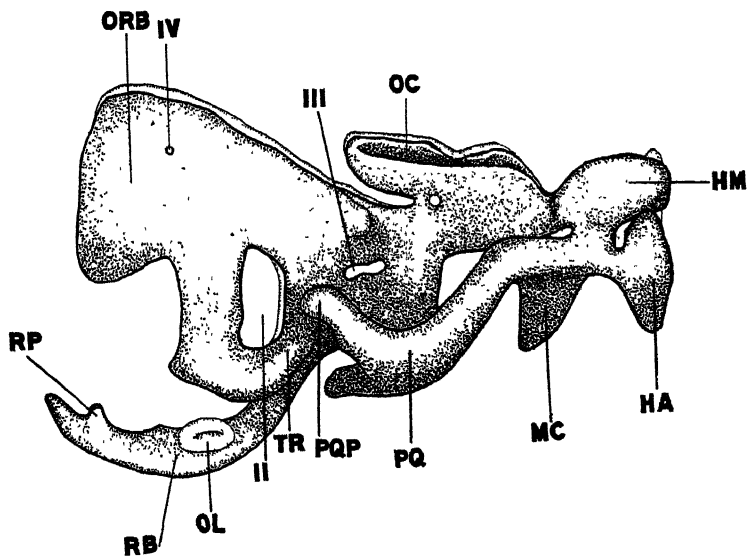


Fig. 11 Left lateral view of the reconstructed chondrocranium, showing relations of orbitals, trabeculae, otic capsule, and mandibular arch; 40-mm. *S. sucklii*.  $\times 16\frac{1}{2}$ .

parachordals a distance equal to the diameter of the notochord in this region. The posterior limit of the otic vesicle is in the same transectional plane as the anterior limit of the vagus nerve. Thus we can say that the otic vesicle extends from the posterior limit of the acousticofacialis to the anterior limit of the vagus. These limits are relatively much greater now than at any previous or subsequent time. The endolymphatic ducts extend dorsally from about the middle of the dorsal walls of the otic vesicles. Each bends caudally and opens on the external surface of the head near the mid-

dorsal line. The opening is approximately in the same trans-sectional plane as the anterior limit of the vagus nerve. The first differentiation to form the floor of the otic vesicles occurs in the 25-mm. embryo, appearing as precartilage forming from the parachordal plate. This is not shown (fig. 5), as only true cartilage was considered in making the model; however, it occurs in the region of the greatest width of the parachordal

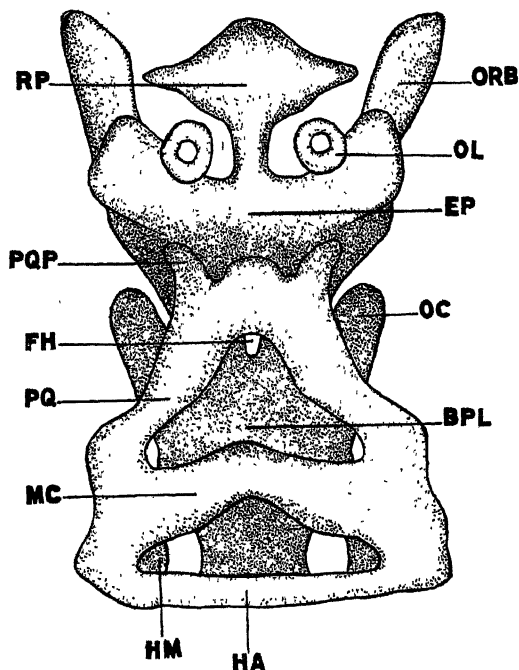


Fig. 12 Ventral view of the reconstructed chondrocranium, showing the relations of the rostral plate, ethmoid plate, and the mandibular arch; 40-mm. *S. sucklii*.  $\times 16\frac{1}{2}$ .

plate. The floor of the otic capsule and dorsal elevations on the parachordals are present at the 35-mm. stage (figs. 7 and 8). These elevations lie median and caudad to the posterior limits of the otic vesicles. Lateral to the otic vesicle is an elevation of the floor of the capsule, which is the beginning of its outer wall. Also, an anterolateral process from the floor of the capsule is present with upturned edges. These latter structures represent the beginnings of the inner and

outer walls of the cephalic portion of the future otic capsule. Thus the inner wall at this stage has two distinct parts, while the outer is represented by only one. van Wijhe ('05) found the first trace of the otic capsule to be independent of the parachordals. The author's findings agree with those of Sewertzoff.

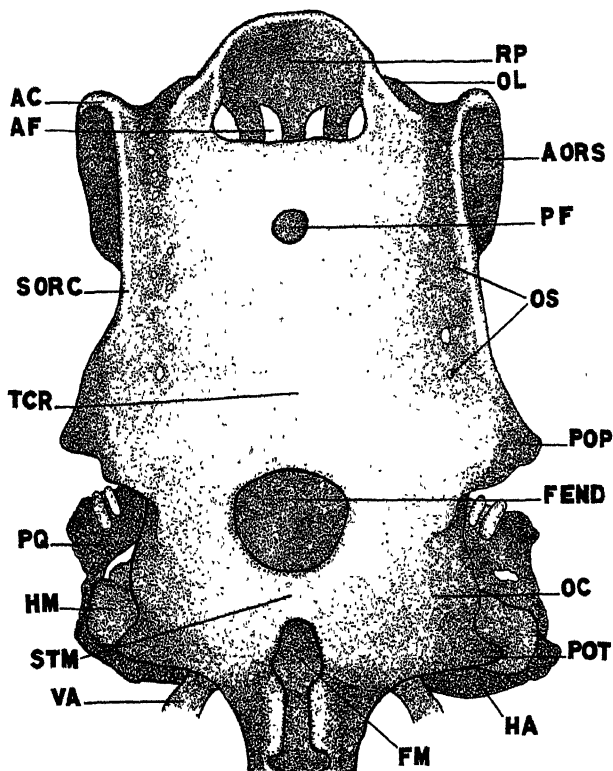


Fig. 13 Dorsal view of the reconstructed chondrocranium, showing the dorsal foramina, supra-orbital crest, and the pre- and postorbital ridges; 60-mm. *S. acanthias*.  $\times 12\frac{1}{2}$ .

The otic capsule in the 40-mm. *S. sucklii* (figs. 10 and 11) is at practically the same stage of development as that of the 35-mm. *S. acanthias*. However, the cartilage of the capsule which is present is thicker and more definitely demarcated.

In the 60-mm. specimen (figs. 13 and 14) the parachordal region is relatively narrower than in the preceding stage, since a considerable part has been utilized in forming the floor of the otic capsule. The basal plate is relatively shorter, as adjacent parts are surpassing it in development. At the posterior end of this region (fig. 14) the notch between the otic capsule and the first occipital vertebra has been closed by the union of the neuropophysis of the vertebra with the

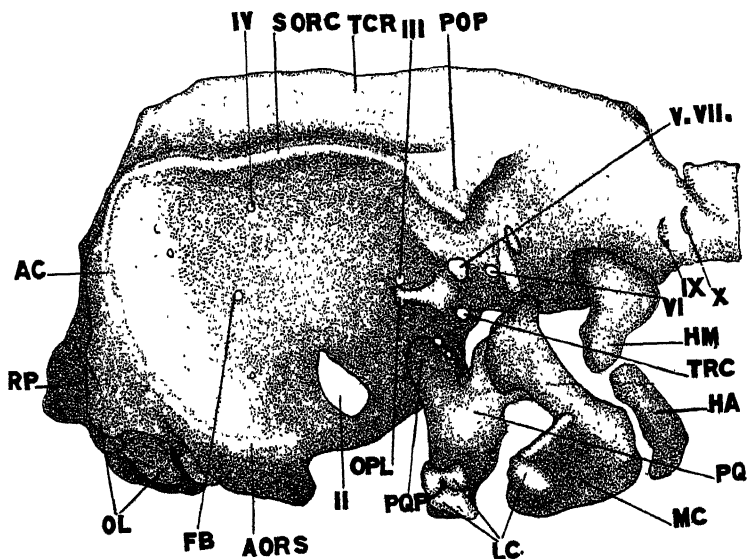


Fig. 14 Left lateral view of the reconstructed chondrocranium, showing the orbit of the eye as outlined by the supra- and antorbital crests, also the optic pedicle, olfactory cartilages, and the first two branchial arches; 60-mm. *S. acanthias*.  $\times 12\frac{1}{2}$ .

posterior end of the capsule, thus completing the foramen for the glossopharyngeal nerve. In a similar way the post-vagus neural arch has joined the structure in front, enclosing the vagus nerve in its foramen. From these structures, as well as from the dorsal margin of the posterior part of the capsules, the synotic tectum has been formed. Externally the capsules are not sharply marked off from the other parts, and at least in this specimen, the elevations of the surface, so noticeable in the adult, as indicating the position of the

various semicircular canals, are scarcely recognizable. The inner wall of each otic capsule is now more nearly completed, there being three openings from the cranial cavity into the labyrinth. The most anterior of these is just below and a little behind the anterior cupola, and above the level of the parachordal plate, which serves as a passage for the auditory nerve into the capsule. In this region there is a curving downward of the roof to form the inner wall of the capsule. A slight distance farther posterior is the foramen for the glossopharyngeal nerve. This foramen extends backward through the lower otic region at an angle of about  $45^{\circ}$  from the main axis of the embryo, and opens on the surface of the caudolateral angle of the otic capsule (fig. 14). The third foramen extending through the inner wall of the capsule is for the passage of the endolymphatic duct, which extends from the sacculus dorsomedially close to the cranial wall in an irregular course to the endolymphatic foramen in the tegmen cranii. There is also a pronounced change in the outer wall from that found in the earlier embryos. It has become much thicker, projecting farther laterally and forming the postorbital process (fig. 13). This outer wall is on the average over twice as thick as the inner, and at this stage contains the horizontal semicircular canal near its external surface. The posterior cupola contains the posterior vertical semicircular canal, which is separated from the utricle along its middle portion by a cartilaginous partition. Outside the partition, between it and the utricle, lies the auditory nerve, and below it the glossopharyngeal. These nerves extend from their inner foramina approximately parallel with one another for a short distance, where the auditory turns medially, passes through the cartilaginous partition, and enters the ampulla of the posterior vertical canal. These nerves thus cross each other as in the adult median to the utricle, near its posterior limit. In comparing the 60-mm. specimen with the adult as described by Miss Wells ('17), the writer concludes that the anterior and posterior cupulas are never directly connected with the basal cranii, but are developed from the anterior

and posterior portions, respectively, of the lateral processes of the basal crania.

#### ORBITALS AND TRABECULAE

The orbitals (alisphenoids) appear as true cartilages in the 27-mm. stage. They are separate rod-like chondrifications extending forward parallel with the notochord, with their posterior ends almost in line with the anterior portion of it. Sewertzoff ('99) described and illustrated the orbitals (alisphenoids) as arising independently of the parachordals. van Wijhe ('05) described them as being an outgrowth of the parachordals. The condition of the orbitals in the 27-mm. stage agrees closely with the account given by Sewertzoff. Undoubtedly, van Wijhe worked on an older specimen, in which the orbitals, after having first arisen free from the parachordals, had later united with them. In the region below the anterior ends of each of the parachordals, the trabeculae also are found for the first time. They normally lie below and parallel to the orbitals, but have become shifted in position, until they extend almost at right angles to the orbitals and also lateral and posterior to the middle of the hypophysis. They are rod-like structures, entirely free from the orbitals, and dip backward slightly near the center. As in previous stages, the forebrain is bent downward and backward and the trabeculae appear to have been carried to their present position by it.

Each orbital of the 35-mm. *S. acanthias* (figs. 7, 8, and 9) may be roughly considered as an acute isosceles triangle, with its base at the anterior end, and with its apex joined to the anterolateral edge of the parachordal, just below the exit of the acustofacialis nerves. The lower edges of the orbitals are in the same horizontal plane as the parachordals, while the upper forms an angle of about  $30^\circ$  with the same. The broad portions of the orbitals later assist in separating the optic from the cranial vesicles. At about the center of the orbital (fig. 8) is a small foramen for the passage of the trochlearis nerve. The anterior fourth of each orbital lies in the

vertical plane, but farther back, the two converge downward, and diverge from the vertical plane, reaching its extreme close to the junction with the basal plate, where the orbitals lie at about an angle of  $45^{\circ}$ . The 'warping' of these plates is in close relation with the varying contours of the adjacent parts of the brain. The orbitals (fig. 7) join the parachordals not at their extreme anterior ends, but about midway between that point and the anterior limit of the base of the floor of the otic capsule. This, no doubt, is the more primitive condition, as the space between the anterior ends of the parachordals and the orbitals soon fills up, giving the appearance indicated in Sewertzoff's figure 13. It is found, as did Sewertzoff, that the notochord has a free anterior end, but he seems to have overlooked the ventrally recurved anterior hook. The orbitals, which are separate formations in his figure 5, are here united to the anterior lateral edges of the parachordals. The parachordals are much thicker near their anterior ends, in the region where the trabeculae join them. Sewertzoff makes no mention of this thickening.

The greatest change at this 35-mm. stage is in the trabeculae. The principal axis of each is still nearly vertical, the two being about parallel to each other. A transverse bar joins the upper extremities of the trabeculae with one another and with the lower surfaces of the parachordals and appears to be continuous with these structures at each of its ends. This is undoubtedly the polar cartilage mentioned by van Wijhe ('22). From their lower ends, the trabeculae have grown forward in nearly a horizontal plane, and the two have expanded, so that they almost immediately fuse to form the ethmoid plate (fig. 9). Thus is outlined the fenestra hypophyseos, bounded dorsally by the dorsal plate and ventrally by the ethmoid plate. The space is about four or five times as long as wide, its major axis being nearly vertical, and the sides converge slightly forward and downward. The junction of the trabeculae and basal plate in the 35-mm. *S. acanthias* seems to be affected in part by a downgrowth from the basal plate, since at the point of union the cartilage is wider



than a little farther ventral along the trabeculae. After a short narrow part, the trabeculae suddenly widen, the beginning of the morphologically upward growth, which is later to cause their union with the orbitals and at the same time contribute to the formation of the cranial wall, which later separates the brain from the optic vesicle. A peculiar feature is the existence of a small transverse bar, extending between the two trabeculae, close beneath the basal plate, separating a short but wide foramen from the fenestra hypophyseos. Through this passes a very small blood vessel, which from its relation is apparently the common carotid of the adult, its two halves being united even at this early stage.

In the 40-mm. *S. sucklii* the orbitals extend anterolaterally from the cephalic ends of the parachordals much the same as in the 35-mm. *S. acanthias*. However, their upper and lower edges have advanced, so that they enclose more of the brain (figs. 8 and 11). Their density has also noticeably increased and their union with the basal plate is more definite over a larger area. The most striking advance of the orbitals is in their relation to the trabeculae. There is now a distinct continuity of cartilage extending downward from the lower proximal edge of the orbitals to the lateral edges of the trabeculae and the ethmoid plate. Also, near the middle lower edge of the orbital, cartilage extends downward and unites with the cornu trabeculae. These newly formed parts more completely separate the telencephalon and diencephalon from the optic vesicle. The large space remaining between these new structures (fig. 11) is the optic foramen. There was no distinct polar cartilage observed in this stage nor in the one following.

In the 60-mm. embryo (figs. 14 and 15), the trabeculae of the two sides have united practically throughout their whole extent, obliterating the fenestra hypophyseos, excepting the small foramen for the common internal carotid artery. On each side the union of the trabecula and orbital has continued, so that now the only gaps between them are the openings for the optic nerve, the oculomotor nerve, and two for

blood vessels entering the cranial cavity, while behind and below the hooked end of the notochord is the small transbasal canal. In this way the inner wall of the definite orbit has been formed. About halfway between the transbasal canal and the oculomotor foramen is the optic pedicle. This con-

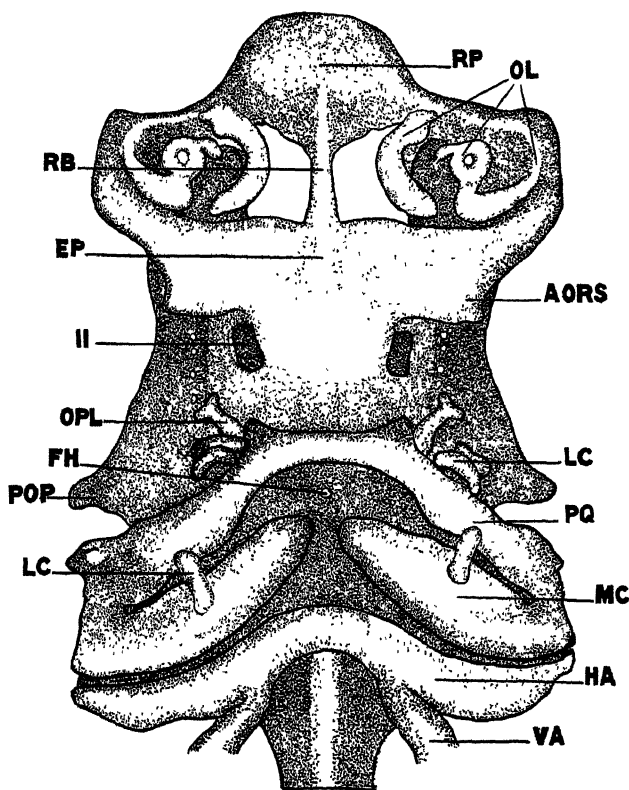


Fig. 15 Ventral view of the reconstructed chondrocranium, showing the rostral plates, olfactory capsule, ethmoid plate, foramen for the optic nerve, and the first two branchial arches; 60-mm. *S. acanthias*.  $\times 12\frac{1}{2}$ .

sists of a cartilaginous rod, continuous with the cranial wall, its distal end expanded like a saucer, to rest against the eye-ball. It is now impossible to ascertain the exact limits of the contributions of orbitals and trabeculae to the cranial wall, except in so far as the line connecting the optic and oculo-motor foramina mark it. Connected with the upper margin

of the orbital part of the wall is the supra-orbital crest (figs. 13 and 14), between which and the tegmen cranii on the dorsal surface is the broad but well-defined groove which is a characteristic feature of the adult cranium. Along the line of this groove (fig. 13) are several foramina for branches going from the superfacialis branch of the facial nerve to the lateral organs on top of the head. These foramina are irregularly arranged and do not correspond accurately in number or position on the two sides. The main trunk of the nerve courses along the upper inner part of the orbit from its entrance with the rest of the seventh to its exit through the upper part of the preorbital process. The supra-orbital crest (fig. 14) is nearly horizontal; in front and behind it passes directly into the pre- and postorbital processes, respectively. Behind, the orbital has now united with the anterior end of the otic capsule, above the exit of the fifth and seventh nerves, but the postorbital process has not yet the extent which it has in the adult. The seventh nerve at its exit and for the greater part of its extent lies wholly within the orbit. None of it is now included in the cartilage in front of the otic capsule.

The openings in the synotic tegmen (fig. 13) are four in number, as follows: 1) The anterior fontanelle, which is structurally more anterior than dorsal in position, extending dorsally from the rostral plate, up and on to the dorsal surface of the brain. It is irregular in outline and it is somewhat difficult to determine the boundary, because the tegmental cartilage becomes very thin, and gradually fades away as it approaches the opening. 2) A short distance behind the anterior fontanelle is the small pineal foramen. It is located above the base of the pineal body rather than above its dorsal extremity as one might suppose. Its exact outline is difficult to determine, since here, as around the anterior fontanelle, the cartilage thins very gradually near the opening. 3) Farther back, at about the level of the middle of the otic capsules, is a large posterior fontanelle in the middorsal line. Its outline is approximately circular and the

cartilage bounding it is relatively thick close to the margin. Through this fontanelle the endolymphatic ducts reach the surface. In later stages a cartilaginous floor will be formed at a lower level across this fontanelle, converting it into the fossa endolymphaticus, with two small openings on either side, the anterior for the endolymphatic duct and the posterior for the perilymphatic duct. 4) The foramen magnum at this stage is relatively large, its dorsal margin being but a short distance behind the posterior fontanelle, the synotic tectum separating the two openings being narrower in the middle line. The foramen magnum extends at least half its vertical diameter above the tops of the neural arches of the anterior vertebrae.

The preorbital crest, the beginning of which in the earlier stage was a discrete cartilage, has joined the anterior orbital wall and is continuous dorsally with the supra-orbital crest. Between it and the orbital wall are already the same foramina which Miss Wells has described in the adult, with practically no differences in their relative positions.

Internally, in the orbital region, the conditions are much as in the adult, except that the hypophyseal pit is not so well defined in front. In its oblique posterior wall is the internal opening of the carotid foramen, while the angle between the trabecular part of the floor and the basal plate forms the dorsum sellae. As stated above, the anterior end of the notochord extends forward beyond the limits of the basal plate, and the internal opening of the carotid foramen lies ventral to and behind its tip. Into this part of the sella turcica the transbasal canal opens. The changes necessary to convert this part into the adult condition are the continued development of cartilage in the sella turcica region, including the tip of the notochord within it.

#### ETHMOID PLATE AND OLFACTORY CAPSULES .

The ethmoid plate in the 35-mm. specimen (figs. 7, 8, and 9) has its longitudinal axis nearly parallel to the basal plate, and consequently at right angles to the greater part of the

trabeculae. It widens immediately at the junction of the two trabeculae, so that its transverse diameter is over twice its length, and is directly below the anterior ends of the parachordals. Its dorsal surface is slightly excavated to accommodate the telencephalon of the brain. At each anterolateral angle it gives off a lateral process. Each expands rapidly in an oblique but nearly vertical plane and the lateral margin is divided by a horizontal groove into a small upper and a much smaller and wider ventral portion. It appears that the dorsal process will contribute to the side wall of the cranium, anterior to the entrance of the optic nerve, and that the lower part of the process is the beginning of the ant-orbital crest, which at the same time forms the posterior wall of the olfactory capsule. These lateral ethmoidal processes have been regarded by some as rudimentary preoral branchial arches. Balfour ('81) regards the exactness of this interpretation as highly improbable, for he says: "The development of these structures as outgrowths of the skull is in itself to my mind a nearly conclusive argument against their being branchial arches, in that two branchial arches hardly ever or perhaps never arise in this way." The results obtained by the writer seem to support Balfour's position. Just ventral to this is a small cartilage, isolated at this time, which participates in forming the wall of the olfactory capsule. It arises as a discrete element, and later becomes associated with cartilages farther forward. In front of the orbital-crest rudiment is another cartilage; it probably forms part of the ventral wall of the capsule. On the ventral side of the ethmoid is a small elevation, the beginning of the median ventral carina. From the anterior margin of the ethmoid (fig. 9) a strong rostral bar extends straight forward for a distance about equal to the length of the ethmoid plate. It is large, strong, and triangular in cross-section. Its dorsal surface is on the level with that of the ethmoid plate, but immediately at its origin it expands downward, so that its lower surface is about on a level with the ethmoid carina. This rostral bar (fig. 7) lies directly below the free end of the notochord and also projects slightly in front of it.

When one compares the lateral views of *S. acanthias* and *S. sucklii* (figs. 8 and 11), respectively, with the lower stages, a noticeable difference is observed in the position of the trabeculae. They no longer lie in a vertical plane to that of the basal plate, as in the 27-mm. stage, but in a plane whose general direction is approximately  $45^\circ$  from that of the basal plate. Thus a partial correction of the displacement of the trabeculae has occurred. The lower ends of the trabeculae have united farther dorsally in the 40-mm. specimen, and the remaining space between them has diminished by the proliferation of precartilaginous cells along their inner edges. This opening which now remains is the fenestra hypophyseos and is in close proximity to the infundibulum and hypophysis. In the 35-mm. stage the space between the lateral anterior vertical portion of the trabeculae includes the infundibulum and the recurved anterior part of the forebrain. This same region (fig. 11) now includes only the infundibulum, the forebrain having been partially straightened. On each side above and in front of the trabeculae, and immediately below the base of the orbital, two large openings persist. These are for the passage of the third cranial nerve to the muscles of the eye, and also for a blood vessel. The ethmoid plate has increased in extent and density until its lateral extensions are now continuous with the lower edge of the proximal portions of the orbitals. The cornua trabeculae are likewise connected with the orbitals (fig. 11), and their part in forming the lower end of the anterior orbital crest at this stage is clearly seen. The rostral plate (figs. 10 and 12) has its dimensions greatly extended over that of the previous stage. It now has processes extending outward and upward in the direction of the lower limits of the orbitals, with which it will ultimately unite, as will be seen in the following stage. The rostral plate now serves an effective support for the anterior portion of the brain, which again suggests that the cranium is primarily for the support of the brain, rather than for its protection. The olfactory capsules (fig. 12) are in an advanced stage of formation in the region surrounded by the

cornu trabecula, ethmoid plate, rostral bar, and rostral plate. The exact limits of these parts are very difficult to determine at this stage, as there is such a gradual transition from typical cartilage cells to precartilaginous cells, which in turn gradually merge into mesenchymal tissue. However, there is a ring of cartilage which is the most highly chondrified portion, surrounding the nasal opening. By the position of the ring of cartilage as well as by the fact that it is not joined to the chondrocranium, it is considered to be primarily for protection of the more delicate nasal structures.

There is nothing in the 60-mm. *S. acanthias* (fig. 15), externally or internally, which serves to draw a sharp line between orbital and ethmoidal regions. In general, it may be said that externally the preorbital process indicates the anterior limit of the trabecular alisphenoid region. Internally, there is in the floor of the distinctly ethmoidal part a shallow depression, a little in front of the optic foramen, which may be the same as the anterior depression in Miss Wells' figure 4. On the ventral surface of the ethmoid region there is still the same carina as in the previous stage, and lateral to this is the lower end of the preorbital process. The rostral bar arising from the anterior margin of the ethmoid plate is still distinct; it is high, narrow, and in front expands laterally into the side walls of the very short rostrum, which shows no evidence of being a compound structure, for there are no traces of the rostral bars of Miss Wells' paper. Laterally and dorsally, the side walls are connected, first, with the wall of the olfactory capsule; more dorsally, with the anterior side of the preorbital process, and above it passes into the general tegmen cranii.

On either side of the rostral bar (fig. 15) is a large fontanelle bounded behind by the ethmoid plate, in front by the rostral wall, and extending laterally into the preorbital plate; the medial part of this fontanelle opens ventrally and persists in the adult. The lateral portion is occupied by the olfactory capsule and will be cut off from the rest by growth of cartilages between the ethmoid plate and the ven-

trolateral side wall of the rostrum. The medial side of the olfactory capsule is formed by a cartilage connected in front with the side wall of the rostrum, while lateral and ventral to it is a cartilage extending forward from the antorbital plate, forming the ventral portion of the capsule. On the lateral and outer half of the anterior side, the capsule is bounded by a downgrowth from the antorbital process which apparently is formed, at least in part, by the discrete cartilage of the 35-mm. stage which then was in front of the antorbital cartilages. This portion bends inward and forward distally and apparently gives rise to the stronger cartilage surrounding the external naris.

#### SUMMARY

1. A series of models showing developmental stages of the chondrocranium were constructed.

2. The parachordals appear as independent precartilaginous areas on each side of, and parallel to, the notochord in the otic region of the 10-mm. *Squalus acanthias*. In the 25-mm. stage chondrification has occurred and they are fused with the notochord, forming the basal plate.

3. The orbitals and trabeculae first appear as independent precartilaginous areas in the 19-mm. embryo, and at the 27-mm. stage are independent cartilages; later, they connect on each side with the basal plate and with each other, forming the cranial wall between the orbital cavity and the brain.

4. An apparent shifting posteriorward occurs in the relative positions of the bases of the fifth, seventh, and eighth cranial nerves in respect to the anterior end of the notochord and otic vesicle from the 10-mm. to the 60-mm. stage.

5. The otic vesicles are proportionately small in the 10-mm. stage and reach their relatively largest size in the 19-mm. embryo.

6. The floors of the otic capsules begin to develop as lateral extensions of the basal plate at the 25-mm. stage. From this anlage chondrification extends to the anterior and posterior cupolas.



7. The neural arches of the cervical region contribute to the formation of the tegmen cranii and the occipital region.

8. The tegmen cranii forms by a chondrification approaching the median plane from each side, and is practically complete at the 60-mm. stage.

9. The head is greatly flexed at the 10-mm. stage, but during subsequent development becomes almost completely straightened.

10. The ventrally recurved anterior end of the notochord is present in the 10-mm. embryo and persists through the 60-mm. stage.

11. The optic pedicle forms as an outgrowth from the lateral surface of the cranial wall, immediately cephalad of the trabecula.

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# POLYMORPHISM AMONG THE SUBGENERA OF NASUTITERMES

L. HARE

FOUR PLATES (EIGHTEEN FIGURES)

## AUTHOR'S ABSTRACT

This study is concerned with the interrelationship existing among the polymorphic soldier castes of six representative subgenera of the genus *Nasutitermes*. By measurements of anatomical parts and camera-lucida drawings, an attempt has been made to compare these particular subgenera on a basis of gradual divergence of certain structures. For this purpose a series of tables was compiled in which apparently unstable structures were compared to a relatively stable figure in all available species of the selected subgenera.

According to my findings, it was concluded that there is no strict correspondence between the major, intermediate, and minor castes of different subgenera of the genus *Nasutitermes*. Particular reference is made to the work of N. Holmgren ('12) and to that of J. S. Huxley on heterogonic growth.

Among those interested in the social insects, the problems of the origin of castes and the relationships existing within the colony have received a great deal of attention. With respect to termites, no theory has yet been offered which has merited universal acceptance. The biological significance of such a study was emphasized by Emerson ('26), special reference being made to the remarkable diversification into two contrasting types of soldiers evolved in *Rhinotermes* s. str.; one is specialized for biting, the other for the emission of a volatile liquid. As material for the present study of the interrelationships which exist among the polymorphic soldier castes of the genus *Nasutitermes*, six subgenera have been used. The number of distinguishable nasute soldiers present varies from one to three; as a group they are characterized (Dudley and Beaumont, '89) by a proboscis-like projection of the head, from the tip of which a viscid, sticky defensive substance is extruded.

Two closely associated problems become involved in this work: first, the relations among individual members of the soldier castes, and, secondly, the relations among the castes

treated as units, the latter suggesting a possible evolutionary sequence. For direction and suggestions in working with this problem and also for the generous loan of his collection of termites, I am very greatly indebted to Dr. A. E. Emerson.

TABLE 1

	DIVERSITERMES SPECIES	VELOCITERMES VELOX
	(Intermediate soldier) 2 individuals	(Major soldier) 1 individual; det. N. Holmgren, Mojos, Bolivia
Total head length	1.34-1.38 mm.	1.41 mm.
Head width anteriorly	.69- .71 mm.	.68 mm.
Head width posteriorly	.71 mm.	.68 mm.
Head thickness anteriorly	.48- .55 mm.	.55 mm.
Head thickness posteriorly	.58 mm.	.58 mm.
Number of antennal joints	14	14
	(Minor soldier) 5 individuals	(Minor soldier) 6 individuals
Total head length	1.23-1.30 mm.	1.27-1.30 mm.
Head width anteriorly	.48- .53 mm.	.41- .52 mm.
Head width posteriorly	.61- .65 mm.	.65- .68 mm.
Number of antennal joints	14	14
Head thickness anteriorly	.39- .44 mm.	.41- .48 mm.
Head thickness posteriorly	.49- .55 mm.	.51- .56 mm.
	MEASUREMENTS OF VELOCITERMES VELOX—HOLMGREN ('10)	
	(Major soldier)	(Minor soldier)
Length of head	1.4-1.49 mm.	1.25-1.39 mm.
Head width	.7- .72 mm.	.65- .74 mm.
Number of antennal joints	14	14
	THE WORKER CASTE	
	Diversitermes species	Velocitermes velox var.
Total head length	1.03-1.23 mm.	.99-1.03 mm.
Head width	.79-1.03 mm.	.82 mm.
Number of antennal joints	15	15
Pronotum width	.44- .58 mm.	.48- .55 mm.
	FROM HOLMGREN ('10) (WORKER)	
Head length	1.2 mm.	
Head width	1.03 mm.	
Number of antennal joints	15	

The specimens, preserved in alcohol, were studied under a 10× ocular with 48-mm. objective, and measurements of all structures considered essential to a comparative study were calculated by means of an ocular micrometer. The number

of specimens from which average figures were compiled varied from two to eight, depending upon availability of material. Holmgren ('10) made a detailed study of the degree of variability encountered in various structural measurements. Head length, for example, he found, upon consideration of from 50 to 128 individuals of several species, did not vary more than 0.2 to 0.4 mm. within any group.

A species of *Diversitermes* (pl. 3) was selected for my use from a colony collected August 14, 1926, by C. Schmidt in Urucum de Corumba, Matto Grosso, Brazil. As this species had not been determined, upon Doctor Emerson's suggestion I compared it with the description and figures of *Velocitermes velox* Holmgren (Holmgren, '10) and also with specimens from the cotype. A consideration of color, pilosity, and measurements led to the conclusion that the larger soldier of *velox* and the intermediate form of the *Diversitermes* species, and likewise the smallest soldiers of each, were similar. A comparison of measurements appears in table 1.

The probability suggested itself that the major soldier of *Velocitermes velox* may have been overlooked when the forms were collected. Matto Grosso, Brazil, is in approximately the same latitude as Mojos, Bolivia, and Chaquimayo, Peru, the regions from which *velox* is recorded. Basing my conclusion upon the comparative evidence cited above, the *Diversitermes* species has been denoted as *velox* in the remainder of this paper.

In the list of the forms studied, which follows, a species of the genus *Armitermes* has been included, since evidence points to the derivation of the genus *Nasutitermes* from *Armitermes* during the course of evolution.

*Armitermes* (*Curvitermes*) *odontognathus* Silvestri

Mojos, Bolivia—det. N. Holmgren

Coll. N. Holmgren

*Nasutitermes* (*Angularitermes*) *nasutissimus* Emerson

Kamakusa, British Guiana—det. A. Emerson

Coll. H. Lang

*Nasutitermes* (*Constrictotermes*) *cavifrons* Holmgren

Kurupung, British Guiana

October, 1922.

Coll. H. Lang

- Nasutitermes* (*Diversitermes*) *velox* Holmgren  
Urucum de Corumba, Matto Grosso, Brazil—det. A. Emerson  
August 14, 1926. Coll. C. Schmidt
- Nasutitermes* (*Tenuirostritermes*) *tenuirostris* Desneux  
Phantom Lake, Texas—det. T. E. Snyder  
Coll. Gage
- Nasutitermes* (*Trinervitermes*) *carbonarius* Sjöstedt  
Faradje, Belgian Congo—det. A. Emerson  
Coll. Lang-Chapin
- Nasutitermes* (*Velocitermes*) *beebei* Emerson  
Kartabo, British Guiana—det. A. Emerson  
October 9, 1920. Coll. A. Emerson

The first question considered was the possible relations existing among the polymorphic soldiers within the castes, namely, whether or not the monomorphic and dimorphic forms were strictly comparable to a particular member of the trimorphic series, or, to express it differently, had these polymorphic soldiers developed in each case so that evolutionary sequence was discernible or did the forms of each subgenus represent an independent type of divergence? In order to form indices which might serve as a test, a constant or stable figure had to be established against which varying structures could be compared; total body length was unsatisfactory because of indefinite shrinkage of the soft parts. Examination of all the structures measured revealed a far greater stability among certain of them than others. Accordingly, measurements of three such structures, pronotum length, pronotum width, and front tibia length were averaged for each form and the resulting figure considered as relatively stable. Four other structures were selected which showed a high degree of variability; these included total head length, nose length measured from tip to anterior edge of antennal fossa, hind tibia length, and antennal joint no. 8 length (pls. 2 and 3). Using variables as dividends and calculated stable figures as divisors, quotients were compiled into series for each species as illustrated in table 2.

Comparisons made of the mono- and dimorphic forms with those of the trimorphic *Diversitermes* forms, in order to test for a possible constant relationship, gave results as indicated

by the hypothetical letters A, B, and C of table 3. These imply that a definite correspondence of forms (calculated on a basis of the indices used) does not exist, excepting the case of *Diversitermes* and *Velocitermes* which is discussed later. The major form was accordingly considered as typical and has been used in subsequent calculations.

Upon this assumption, the figures of each column of table 2 were arranged in serial order, the actual quotients being replaced by the numbers 1 to 7. This is shown in table 4, in which the four columns have been designated as a, b, c, and d, respectively.

TABLE 2

	NOSE LENGTH	HEAD LENGTH	HIND TIBIA LENGTH	ANTENNAL JOINT NO. 8
	'Average'	'Average'	'Average'	'Average'
<i>Curvitermes</i>	1.43	3.36	2.10	.19
<i>Diversitermes</i>	.77-.75-.94	2.34-2.36-2.49	2.53-2.56-2.62	.31-.22-.37
<i>Angularitermes</i>	1.90	3.22	2.50	.31
<i>Tenuirostritermes</i>	1.08	3.08	2.26	.30
<i>Constrictotermes</i>	.93	2.12	2.78	.34
<i>Velocitermes</i>	.89-1.32	2.25-2.66	2.55-2.62	.25-.42
<i>Trinervitermes</i>	1.64-1.61	3.46-3.11	2.42-2.71	.26-.27

TABLE 3

	NOSE LENGTH			HEAD LENGTH			HIND TIBIA LENGTH			ANTENNAL JOINT NO. 8		
	'Average'			'Average'			'Average'			'Average'		
<i>Diversitermes</i>	A	B	C	A	B	C	A	B	C	A	B	C
<i>Angularitermes</i>			C			C	A			A		
<i>Tenuirostritermes</i>			C			C	A			A		
<i>Constrictotermes</i>			C	A					C	(A)		(C)
<i>Velocitermes</i>		B	C		B	C		B	C		B	C
<i>Trinervitermes</i>	A	B			?		A		C	A		C

TABLE 4

	1 = smallest quotient		7 = smallest quotient	
<i>Curvitermes</i>	5	6	7	7
<i>Diversitermes</i>	1	3	2	(3)
<i>Angularitermes</i>	7	5	4	(3)
<i>Tenuirostritermes</i>	4	4	6	5
<i>Constrictotermes</i>	3	1	1	1
<i>Velocitermes</i>	2	2	3	2
<i>Trinervitermes</i>	6	7	5	6

Figures in parenthesis are equal.

Considering each of these columns separately, the results in a general way imply the following associations:

*Nose length.* Diversitermes, Velocitermes, Constrictotermes, and Tenuirostritermes show fairly close relations with relatively short noses.

*Total head length.* Constrictotermes, Velocitermes, and Diversitermes have shorter heads in contrast to the more elongated ones of the other four species.

*Hind tibia length.* Curvitermes, Tenuirostritermes, and Trinervitermes have relatively short legs; Angularitermes approaches the size of Velocitermes and Diversitermes, and Constrictotermes shows unusually long legs.

*Antennal joint no. 8 length.* The grouping here is not very pronounced, though Curvitermes and Trinervitermes show more decidedly short joints, while Constrictotermes falls at the opposite extreme.

To test further the possible significance of these data, in so far as material was at hand, all the other species of these six subgenera were likewise measured and treated in exactly the same manner. The four types of comparison are again designated as a, b, c, and d, respectively, with non-conformities indicated by parentheses.

(Constrictotermes)

cyphergaster Silvestri .....	a	b	(c)	(d)
latinotus Holmgren .....	a	b	c	d

(Diversitermes)

castaniceps Holmgren .....	(a)	(b)	(c)	(d)
diversimilis Silvestri .....	(a)	(b)	(c)	(d)
melanocephalus Snyder .....	a	b	c	(d)

(Tenuirostritermes)

briciae Snyder .....	a	b	c	d
cinereus Buckley .....	a	b	c	d
incisus Snyder .....	a	b	c	d
laticephalus Snyder .....	a	b	c	d

(Trinervitermes)

bettonianus Sjöstedt .....	a	b	c	d
dispar Sjöstedt .....	a	b	c	d
erythrae Holmgren .....	a	b	(c)	d
ibidanicus Sjöstedt .....	a	b	c	d
lutzi Emerson .....	a	b	c	d
oeconomus Tragardh .....	a	b	c	d
trinervius Rambur .....	a	b	c	d

(Velocitermes)

heteropterus Silvestri .....	a	b	c	d
uniformis Snyder .....	a	b	c	d

An additional consideration of characters not indicated numerically included:

1. *Mandible form* (pls. 2 and 3). Trinervitermes—primary tooth either lacking or much abbreviated. Angularitermes—primary tooth distinctly elongated.

2. *Head constriction* (pls. 2 and 3). Trinervitermes—constriction lacking in major soldiers and practically imperceptible in minor. Other subgenera show obvious constriction.

3. *Geographical distribution* (pl. 4). Trinervitermes is the only subgenus having polymorphic soldiers occurring outside the Nearctic or Neotropical regions.

Summarizing these possible relationships, they become more apparent when graphically illustrated by a figure such as plate 1.

A study of the imago caste was approached in essentially the same manner, but the results were not considered as significant, due to the far greater stability of structures in these forms. This caste has apparently undergone only slight structural divergence during the course of evolution.

#### DISCUSSION

Trinervitermes is probably the most difficult subgenus to place, regarding affinities, in such a group. The question must be weighed as to whether the non-measurable factors, as previously discussed, preclude the intimated relationship with Angularitermes and Tenuirostritermes, or whether Trinervitermes may have become geographically separated from the other subgenera quite early and subsequently have evolved along a different line, developing polymorphic soldiers, reduced mandibles, and a more rounded head.

My supposition that there is no strict correspondence between the soldiers of one subgenus and specific members of another subgenus is not in accord with Holmgren's view. Holmgren ('12) suggested definite relationships between the major, intermediate, and minor forms of Diversitermes and members of the other subgenera, but gave no definite basis for his assumptions. The only correspondence I found was between Velocitermes major and minor and Diversitermes



velox intermediate and minor forms. These two are possibly very closely related. Attention should be called to the two species of *Diversitermes*, namely, *diversimilis* and *castaniceps*, which did not conform with *velox* and *melanocephalus*, as was previously indicated. The possibility that this *Diversitermes* subgenus is made up of more widely separated species than is the case with the other subgenera was implied by Snyder ('26). To quote from his paper in which he described *melanocephalus*: "It apparently occupies an intermediate position between Holmgren's subgenera *Diversitermes* and *Velocitermes* and this and other data rather indicate that these subgenera are not valid."

An application of J. S. Huxley's heterogony theory of disharmonically growing organs was attempted with the termites. This hypothesis does not seem to explain the polymorphism among the nasute soldiers. It is not possible to use the total body-length measurements for the comparisons, as Huxley did with *Xylotrupes*, *Camponotus*, and *Uca*, but other figures, substituted, indicate certain discrepancies. Considering the trimorphic *Diversitermes*, the following relationships occur:

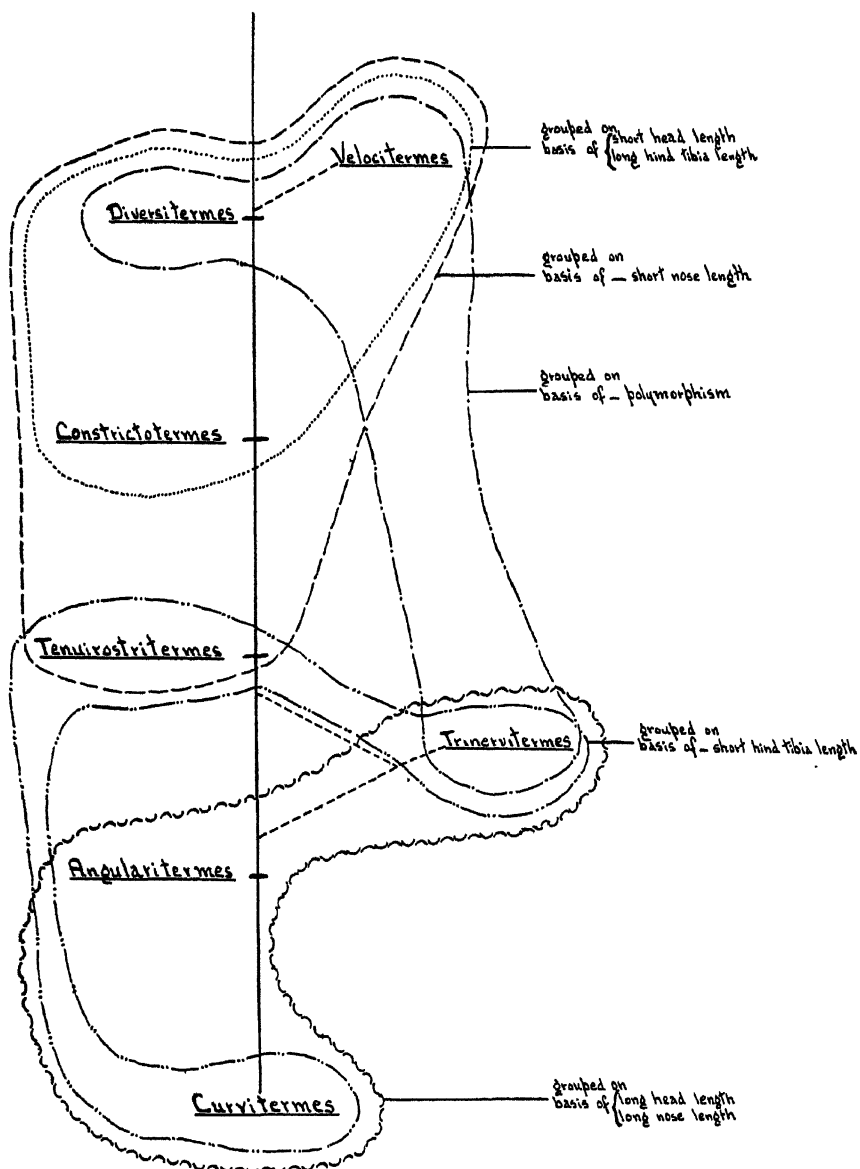
	Major Mm.	Intermediate Mm.	Minor Mm.
Nose length,	.49	.44	.48
Total head length,	1.48	1.37	1.27
Pronotum length,	.24	.24	.17
Pronotum width,	.56	.52	.43

At least superficially, these data do not seem to correlate with Huxley's examples; qualitative as well as quantitative differences are indicated.

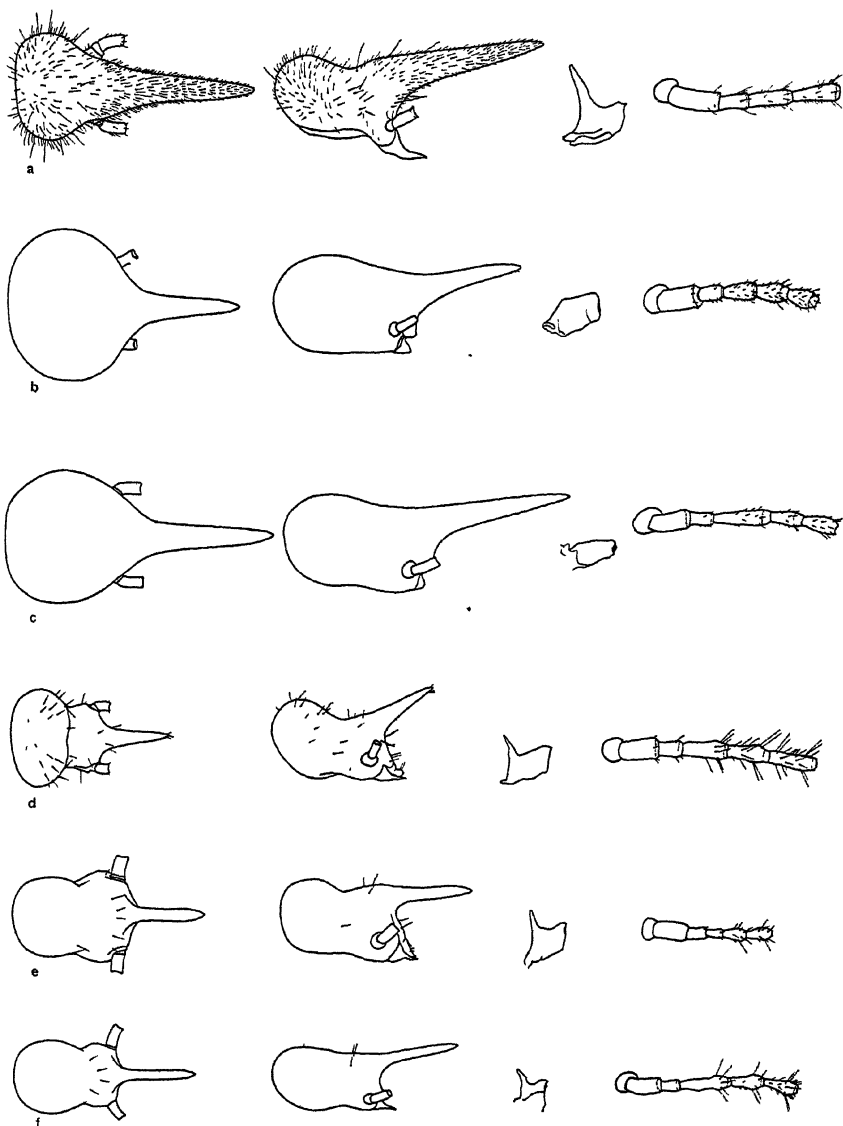
These six subgenera constitute only a fraction of the total number of *Nasutitermes* forms, but the solution of a complete taxonomic problem is often dependent on experimentation with methods of approach to fragmentary portions of it. The more systematically a group of related forms are compared, the greater should be the significance of the result.

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Graphic illustration of possible subgeneric interrelationships.



Soldiers of *Angularitermes*, *Trinervitermes*, *Constrictotermes*, and *Velocitermes*.

Dorsal and lateral views of head, view of left mandible, and view of basal antennal joints of:

- a Monomorphic soldier of *Angularitermes nasutissimus*.
- b Major soldier of *Trinervitermes carbonarius*.
- c Minor soldier of *Trinervitermes carbonarius*.
- d Monomorphic soldier of *Constrictotermes cavifrons*.
- e Major soldier of *Velocitermes beebei*.
- f Minor soldier of *Velocitermes beebei*.

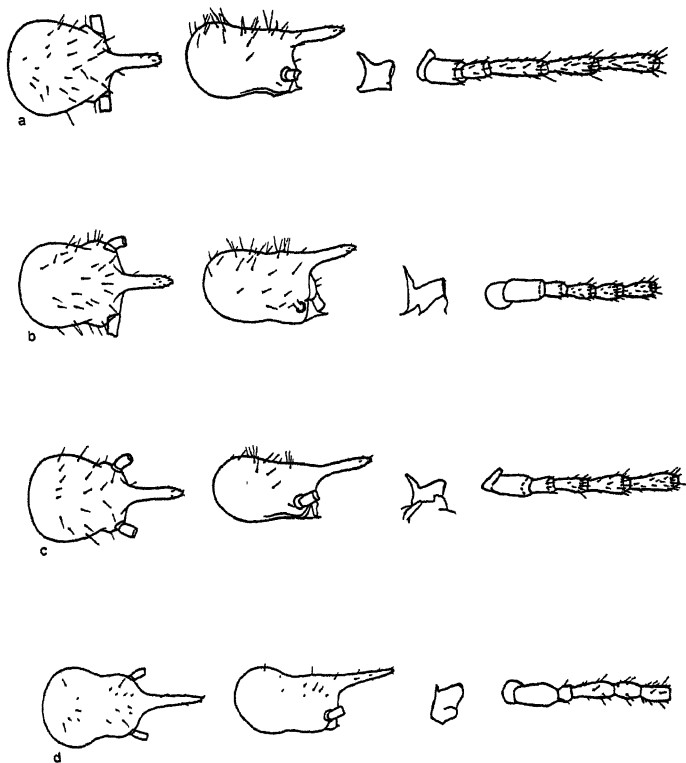
### PLATE 3

#### EXPLANATION OF FIGURES

##### Soldiers of *Diversitermes* and *Tenuirostritermes*

Dorsal and lateral views of head, view of left mandible, and view of basal antennal joints of:

- a Major soldier of *Diversitermes velox*.
- b Intermediate soldier of *Diversitermes velox*.
- c Minor soldier of *Diversitermes velox*.
- d Monomorphic soldier of *Tenuirostritermes tenuirostris*.



## PLATE 4

### EXPLANATION OF FIGURES

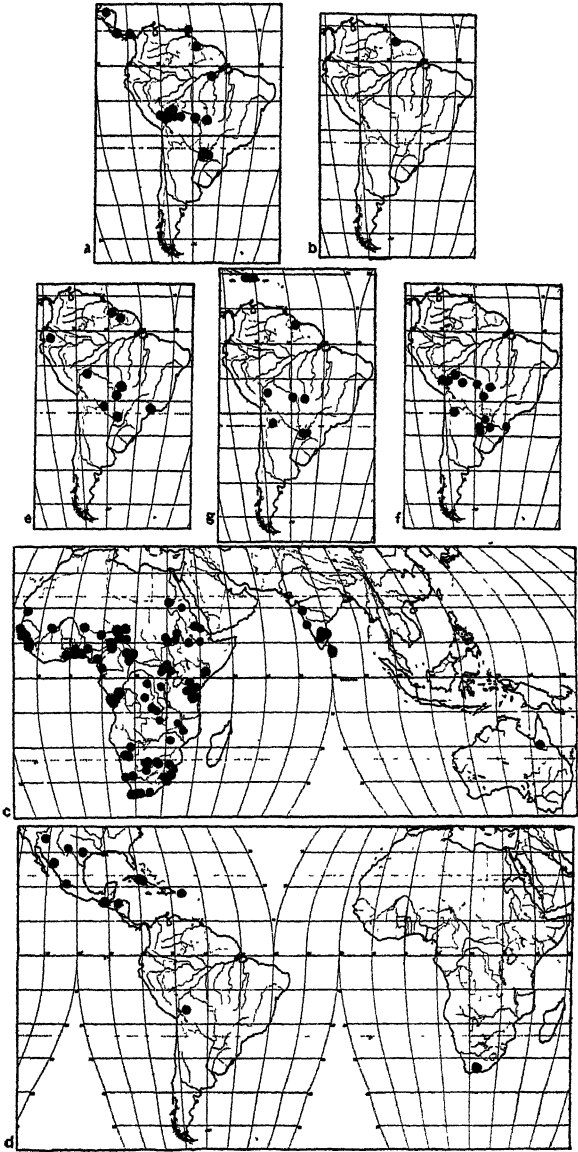
#### Geographical distribution<sup>1</sup>

Plotting of geographical distribution of:

- a Armitermes genus.
- b Angularitermes subgenus.
- c Trinervitermes subgenus.
- d Tenuirostritermes subgenus.
- e Constrictotermes subgenus.
- f Diversitermes subgenus.
- g Velocitermes subgenus.

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<sup>1</sup>The exact location of the following could not be definitely determined:  
a) Rio Madidi and San Fermin, Bolivia; Llinquipata and Putymayo, Peru.  
c) Rohan-Chabot Mission, Angola; Katchinoa, Abyssinia; Bir Joghnan and Gultan, Anglo-Egyptian Sudan; Loubetsi, French Congo; Balandougou, Niger River; Villa Fontes, Zambesi. f) Espia, Canamina, and St. Helena, Bolivia. g) San Bernardino, Brazil.







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